

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/75, 15/74, 15/70, A61K 47/48, 35/74, C07K 14/54		A1	(11) International Publication Number: WO 96/11277 (43) International Publication Date: 18 April 1996 (18.04.96)
(21) International Application Number: PCT/EP95/03921 (22) International Filing Date: 4 October 1995 (04.10.95) (30) Priority Data: MI94A002025 5 October 1994 (05.10.94) IT (71) Applicant (for all designated States except US): DOMPE' S.P.A. [IT/IT]; Via Campo di Pile, I-67100 L'Aquila (IT). (72) Inventors; and (75) Inventors/Applicants (for US only): TAGLIABUE, Aldo [IT/IT]; Via dei Guelfi, 5, I-67100 L'Aquila (IT). BORASCHI, Diana [IT/IT]; Via dei Guelfi, 3, I-67100 L'Aquila (IT). BOSSU', Paola [IT/IT]; Via F.P. Michetti, 4, I-67100 L'Aquila (IT). MACCHIA, Giovanni [IT/IT]; Via Monte Velino, 57, I-67051 Avezzano (IT). MAURIZI, Giovanni [IT/IT]; Via Alba Fucens, 6, I-67100 L'Aquila (IT). PORZIO, Stefano [IT/IT]; Via Pratelle Vetoio, I-67100 L'Aquila (IT). RUGGIERO, Paolo [IT/IT]; Via Teramana, 3, I-67100 Pizzoli (IT). (74) Agent: MINOJA, Fabrizio; Studio Consulenza Brevettuale, Via Rossini, 8, I-20122 Milano (IT).		(81) Designated States: AL, AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MX, NO, NZ, PL, PT, RO, RU, SE, SG, SI, SK, TJ, TM, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	

(54) Title: MICROORGANISMS AS THERAPEUTIC DELIVERY SYSTEMS

(57) Abstract

Methods and compositions for delivery of therapeutic compounds to an animal by administration of a recombinant bacterium to the animal, the bacterium encoding the therapeutic protein.

P. ... 72
USP 45 ...

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Larvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

MICROORGANISMS AS THERAPEUTIC DELIVERY SYSTEMS

FIELD OF THE INVENTION

The invention relates to the use of microorganisms as vehicles for delivery of therapeutic compounds to animals.

5 BACKGROUND OF THE INVENTION

The advent of compounds which are generated using recombinant DNA technology has facilitated development of a vast array of therapeutic agents having the potential to treat a great variety of disease states in
10 animals, in particular in humans. These agents are primarily protein in nature.

While recombinant proteins represent one of the most promising groups of therapeutic agents since the discovery of antibiotics, several problems accompany
15 their use. In particular, systemic administration of such proteins to a animal almost always evokes an immune response which may result in destruction of the protein. Thus, the fate of many recombinant proteins, obtained following laborious and costly procedures, may be rapid
20 destruction in the animal by proteolytic mechanisms. The aforementioned difficulties may be exacerbated in situations in which the target site for the action of the drug is the mucous membranes of the animal which are known sites of enhanced proteolysis. Such mucous
25 membrane surfaces include the oral/intestinal tract, the bronchial/nasopharyngeal tract and the reproductive system in the animal.

Local administration of recombinant protein drugs circumvents some of these difficulties by facilitating

CONFIRMATION COPY

enhanced efficacy of the protein and a diminution in the catabolism thereof. However, there is a paucity of methods for local administration of drugs into areas such as the intestine, the bronchial tract and even the reproductive system.

It has now been surprisingly found that the in vivo administration of a *B. subtilis* engineered with IL-1ra gene results in detectable plasma levels of the expressed IL-1ra, showing therefore the possibility of a trans-mucosal absorption of proteins having pharmacological activity.

This finding opens therefore new therapeutic possibilities by using *per se* known transformed microorganisms, until now described and used for the fermentative production of bulk recombinant proteins, as carriers for the in vivo release of said drugs having protein structure.

Most of the microorganisms until now used for the production of recombinant proteins can be in fact safely administered to humans and animals, being usual components of the physiological flora or being devoid of any pathogenetic risk. This is particularly true for *Bacillus subtilis* which has been widely used as a cloning vector for producing a large number of eukaryotic proteins (Microbiol. Rev. 1993, 57:109) in view of its recognized advantageous properties.

Other species, already used for the production of recombinant proteins, can also be used provided they meet the requisites of non-pathogenicity and ability to colonize human or animal mucosae. The present invention allows therefore, by suitably selecting and adapting the

microorganism, the protein to be expressed and the expression vectors, previously used for the production in laboratory or industrial environment, to address specific therapeutic problems.

5 The present invention concerns therefore the therapeutic use of said engineered microorganisms and compositions containing the same, thereby satisfying a long felt need in the area of therapeutic delivery of drugs to an animal.

10 PRIOR-ART

 The in vivo administration of genetically engineered microorganisms has been already proposed as a means to induce immunization against antigens of pathogenic microorganisms. In this case the gene coding
15 for protective antigens has been suitably inserted into bacterial DNA.

 Examples of said antigens comprise HIV proteins or fragments thereof (WO 92/21376, Nature, Vol. 351, p. 479-482, 1991), B. anthracis toxins (WO 92/19720), E7
20 protein from human papilloma virus type 16 (Infection and Immunity, 60, 1902-1907, 1992), binding region BB of streptococcal protein G and a protein fragment from human Rous Sarcoma Virus (Gene, 128, 89-94, 1993).

 In most cases, these antigens are expressed on the
25 cell surface of the transformed microorganism from which they are not released. The microorganism act therefore as a carrier and adjuvant for the selected antigen which preferably should not be released in order to exert their immunogenic and vaccinogenic activity.

30 This approach, which could be defined as vaccinological approach, has been up to now exploited

using *Staphylococcus xylosus*, *Bacillus anthracis*, *Streptococcus pyogenes* or *Mycobacteria* (BCG) strains.

In contrast to the rather large number of reports concerning the use of genetically engineered microorganisms as vaccines, to the best of our knowledge there is only one report suggesting that a vaccine strain of *Salmonella typhimurium* could be engineered to deliver therapeutic proteins in vivo (J. Immunol., 148, 1176-1181, 1992).

However, the results obtained using human IL-1 β as recombinant protein were disappointing since, in the considered experimental model, only the intravenous administration of the transformed bacteria gave an adequate protection whereas the oral or the i.p. route of administration gave inconsistent protection even at a high dose range. Since the practical possibility of injecting I.V. living bacteria in humans is ruled out, this report, rather than suggesting the possibility of a mucosal biodelivery by administration of engineered microorganism, actually teaches away from our approach.

SUMMARY OF THE INVENTION

The invention refers to pharmaceutical compositions containing, as the active principle, engineered microorganisms expressing non-vaccinogenic pharmacologically active recombinant therapeutic proteins, wherein said microorganism is not *Salmonella* species.

DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of the IL-1ra expressing plasmid, pSETA-IL-1ra.

Figure 2 is a schematic diagram of the plasmid

pSM441.

Figure 3 depicts a densitometric analysis of proteins following SDS-PAGE of IL-1ra-containing samples obtained from *Bacillus subtilis* expressing the same. 1: Molecular weight (in kDa) markers obtained from Bio-Rad (15 μ l); 2: Cell lysate supernatant (15 μ l); 3: partially purified IL-1ra obtained from cell lysate supernatant (15 μ l); 4: Sporulation supernatant (15 μ l); 5: partially purified IL-1ra from sporulation supernatant (15 μ l). The arrows indicate the electrophoretic migration of IL-1ra.

Figure 4 is the nucleotide and corresponding amino acid sequence of human IL-1ra. The numbering of the amino acid sequence (in one letter code) refers to the complete protein including the signal peptide (first 25 amino acids). The mature protein begins at the amino acid arginine, amino acid number 26 (R26).

Figure 5, comprising parts A, B and C depicts in (A) a schematic representation of the life cycle of *Bacillus subtilis* including sporulation; in (B) there is shown a photograph of a Western blot experiment detecting the presence of IL-1ra in the sporulating supernatant of *Bacillus subtilis*. Lane 1, wild type *Bacillus subtilis*; lane 2, *Bacillus subtilis* comprising pSM539 and expressing recombinant IL-1ra; in (C) there is shown a graph depicting detection of IL-1ra in a sporulation supernatant as assessed by SDS-PAGE and laser scanning densitometry.

Figure 6 is a graph depicting the serum concentration of IL-1ra in rabbits administered a single intracolonic instillation of 2×10^9 live *Bacillus*

subtilis per rabbit comprising pSM539 or pSM214. IL-1ra was assessed by ELISA.

Figure 7 is a graph depicting the serum concentration of IL-1ra in rabbits administered two intracolonic instillation of 2×10^9 live *Bacillus subtilis* comprising pSM539 or pSM214. IL-1ra was assessed by BIAcore. Each circle represents an individual rabbit.

Figure 8 is a graph depicting the increase in body temperature of rabbits treated intravenously with recombinant human IL-1 β at 75 ng/kg one hour following a single intracolonic instillation of 2×10^9 live *Bacillus subtilis* per rabbit comprising pSM539 or pSM214.

Figure 9 is a graph depicting increase in body temperature of rabbits receiving a single intracolonic instillation of 2×10^9 live *Bacillus subtilis* per rabbit comprising pSM261 or pSM214. In the group treated with IL-1 β expressing bacteria, 40% of the animals died of hypotensive shock.

Figure 10 is a graph depicting hypoglycemia induced by IL-1 β administered intraperitoneally at a dose of 100 ng/mouse. The animals were also administered subcutaneously either saline (filled bar) or 100 μ g of IL-1ra (cross-hatched bar) or 5×10^8 live *Escherichia coli* comprising pT7MILRA-3 (hatched bar) each of which was administered 2 hours prior to IL-1 β treatment.

DETAILED DESCRIPTION

The present invention relates to the preparation of pharmaceutical compositions comprising recombinantly engineered microorganisms, which microorganisms are

useful for therapeutic treatment of a variety of disease states in animals. The invention further includes methods of administration of such pharmaceutical compositions to an animal having a disease state requiring treatment with the pharmaceutical compositions of the invention.

The invention includes pharmaceutical compositions comprising recombinantly engineered microorganisms which are useful for therapeutic treatment of various pathological conditions. The methods of the invention exploit the ability of certain microorganisms to survive in the mucosal surfaces of animals, which mucosae represent the interface between the exterior and interior regions of the body, and/or undergo sporulation or lyse and release the recombinant proteins expressed at said mucosal surfaces. Once administered to the animal, the recombinant microorganisms of the invention which encode the desired therapeutic protein, express and produce the same. The protein so produced then has the desired therapeutic effect either at the site of production, or is selectively transported to the desired anatomical site at which it then exerts the desired therapeutic effect.

In the present invention, microorganisms are manipulated to express desired recombinant proteins, which microorganisms have properties which render them particularly useful for generation of the compositions of the invention and in the methods of their use. Certain properties of bacteria and other microorganisms are exploited in order to render them useful as vehicles for administration of therapeutic recombinant proteins

to animals. These properties include, but are not limited to, the ability of the microorganism to adhere to epithelial cells (Karlsson et al, 1989, Ann. Rev. Biochem. 58:309); microorganisms which form spores which are resistant to adverse conditions and which are capable of producing large quantities of proteins (Kaiser et al., Cell 1993, 73:237).

Microorganisms are known to possess selective tropism for the mucosa of the intestinal tract, the mucosa of the mouth and oesophagus, the mucosa of the nose, pharynx, trachea, the vaginal mucosa, the skin, the bronchopulmonary system, the eye, the ear.

Specifically, according to the methods of the invention, a microorganism, preferably a bacterium, is manipulated such that it comprises a desired gene, which gene encodes a desired protein useful for treatment of a particular disease state in an animal. The microorganism is administered to the animal following which administration, the protein is produced therefrom to provide the desired therapeutic treatment in the animal.

In addition to comprising the desired gene, the microorganism may also be manipulated to encode other sequence elements which facilitate production of the desired protein by the bacterium. Such sequence elements include, but are not limited to, promoter/regulatory sequences which facilitate constitutive or inducible expression of the protein or which facilitate overexpression of the protein in the bacterium. Additional sequence elements may also include those which facilitate secretion of the protein from the bacterium, accumulation of the protein within the

bacterium, and/or programmed lysis of the bacterium in order to release the protein from the same. Many of the sequence elements referred to above are known to those skilled in the art (Hodgson J., 1993, Bio/Technology 11:887).

For example, heat induction, galactose induction, viral promoter induction and heat shock protein induction systems are well described in the art and are readily understood by those skilled in the art. Additional inducible expression systems include gene expression systems which respond to stress, metal ions, other metabolites and catabolites. Other elements which may be useful in the invention will depend upon the type of bacterium to be used, the type of protein to be expressed and the type of target site in the animal. Such elements will be readily apparent to the skilled artisan once armed with the present disclosure.

This invention includes microorganisms which are capable of producing a pharmacologically active protein. The pharmacologically active protein may be produced within the microorganism and be released upon lysis of the same: the protein may be excreted by the microorganism, or may be released by the microorganism upon sporulation, or upon germination of the spore to form a vegetative cell, or upon lysis.

The types of microorganisms which are useful in the invention include, but are not limited to, yeast and bacteria. Yeast microorganisms suitable in the invention include, but are not limited to, *Hansenula polymorpha*, *Kluiveromyces lactis*, *Kluiveromyces marxianus* subspecies *lactis*, *Pichia pastoris*, *Saccharomyces cerevisiae* and

Schizosaccharomyces pombe.

Bacterial microorganisms suitable for use in the invention include, but are not limited to, Bacillus subtilis and other suitable sporulating bacteria; members of the genus Bifidobacterium including but not limited to, Bifidobacterium adolescentis, Bifidobacterium angulatum, Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium catenulatum, Bifidobacterium infantis, Bifidobacterium longum, and Bifidobacterium pseudocatenulatum; members of the genus Brevibacterium including but not limited to, Brevibacterium epidermis and Brevibacterium lactofermentum; members of the genus Enterobacter including but not limited to, Enterobacter aerogenes, Enterobacter cloacae; members of the genus Enterococcus including but not limited to Enterococcus faecalis; members of the genus Escherichia, including but not limited to, Escherichia coli; members of the genus Lactobacillus including but not limited to, Lactobacillus acidophilus, Lactobacillus amylovorus, Lactobacillus bulgaricus, Lactobacillus brevis, Lactobacillus casei, Lactobacillus crispatus, Lactobacillus curvatus, Lactobacillus delbrueckii, Lactobacillus delbrueckii subspecies bulgaricus, Lactobacillus delbrueckii subspecies lactis, Lactobacillus fermentum, Lactobacillus gasseri, Lactobacillus helveticus, Lactobacillus hilgardii, Lactobacillus jensenii, Lactobacillus paracasei, Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus reuterii, Lactobacillus sake and Lactobacillus vaginalis; members of the genus

Lactococcus including but not limited to, Lactococcus lactis, Lactococcus lactis subspecies cremoris and Lactococcus lactis subspecies lactis; members of the genus Propionibacterium including but not limited to
5 Propionibacterium jensenii; members of the genus Staphylococcus including but not limited to, Staphylococcus epidermidis; members of the genus Streptococcus, including but not limited to, Streptococcus lactis, Streptococcus faecalis,
10 Streptococcus gordonii, Streptococcus pyogenes, Streptococcus mutans, Streptococcus thermophilus and Streptococcus salivarius subspecies thermophilus.

Preferably, the microorganism of the invention is a bacterium. More preferably, the microorganism is an
15 enteric microorganism or a member of the genus Bacillus, particularly B. subtilis and, even more preferably, the microorganism of the invention is either a member of the genus Lactobacillus or the organism is Bacillus subtilis encoding recombinant interleukin 1 receptor antagonist
20 (IL-1ra).

Examples of other microorganisms which are useful in the invention include Streptococcus pyogenes, Streptococcus mutans or Streptococcus gordonii, each being capable of colonizing the oral mucosa and
25 expressing and releasing an anti-inflammatory protein capable of ameliorating inflammatory diseases of the gums and teeth.

Similarly, it is possible to exploit the ability of, for example, Escherichia coli, to colonize the
30 intestinal mucosa in order to introduce therapeutic proteins into this region of the body for treatment of

intestinal disease including among others for example, ulcerative colitis and Crohn's disease. Such bacteria may be administered to the animal either orally or rectally. In view of the high absorption capacity of the intestinal mucosa, according to the methods of the invention, expression of recombinant proteins by recombinant bacteria in the intestinal mucosa can result in transport of the produced recombinant protein across the mucosal surface into the bloodstream. Thus, systemic delivery of recombinant proteins is also contemplated by the invention using recombinant microorganisms capable of expressing the same.

It is also possible to use spore-forming bacteria (i.e., Clostridium and Bacillus) which, when in spore form, are naturally resistant to extreme environments and are therefore particularly suitable for oral administration as they are resistant to the effects of gastric acids. Such bacteria, when administered orally to an animal, should reach the intestinal mucosa in an intact, unchanged state. Upon germination, these bacteria then produce the desired active protein in the intestinal mucosa, which protein otherwise may not have survived the effects of the gastric acids.

Spore-forming bacteria may be additionally exploited for their ability to produce spores and thereby deliver proteins to target mucosal sites in the body. In this instance, vegetative state spore-forming bacteria encoding the desired protein are prepared in a formulation suitable for oral or rectal administration. Upon reaching the intestinal mucosa, such organisms are induced to sporulate wherein the vegetative cells lyse

thereby releasing the expressed protein into the mucosa. In this manner, a well defined dose of the desired protein is released into the mucosa. Induction of sporulation by bacteria in the intestine or induction of germination of spores is accomplished by further manipulating the genes of these organisms which control such events. Importantly, spore-forming bacteria may be engineered such that they are induced to initiate the process of sporulation but are incapable of forming spores. In this case, the cells containing the desired expressed protein lyse thereby releasing the protein; however, since spores are not in fact formed, no live bacteria remain in the host.

The therapeutic protein, the gene of which is inserted into a suitable expression vector, must meet the following requisites in order to be effectively used:

- it must be non-toxic and non-pathogenic;
- it must be non-vaccinogenic, i.e. it should not induce a significant immune response which is protective for the host against the protein itself;
- it must be active in the expressed form or, at least, it must be converted into the active form once released by the microorganism.

Examples of proteins which may be administered according to the invention are mostly eukaryotic proteins, particularly those the expression of which in *B. subtilis* has been disclosed in Microbiol: Rev. 1993, 57:109. More particularly, genes encoding proteins which are useful in the invention as recombinant therapeutic proteins include, but are not limited to, the following

genes. Members of the interleukin family of genes, including but not limited to IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14 and IL-15 and genes encoding receptor antagonists thereof. Genes which encode hemopoietic growth factors, including but not limited to, erythropoietin, granulocyte colony stimulating factor, granulocyte macrophage colony stimulating factor, macrophage colony stimulating factor, stem cell factor, leukaemia inhibitory factor and thrombopoietin are also contemplated in the invention. Genes encoding neurotropic factors are also contemplated, including but not limited to, nerve growth factor, brain derived neurotropic factor and ciliary neurotropic factor. In addition, genes which encode interferons, including but not limited to IFN-alpha, IFN-beta and IFN-gamma are included. Further contemplated in the invention are genes encoding chemokines such as the C-C family and the C-X-C family of cytokines, genes encoding hormones, such as proinsulin and growth hormone, and genes encoding thrombolytic enzymes, including tissue plasminogen activator, streptokinase, urokinase or other enzymes such as trypsin inhibitor. The invention further includes genes which encode tissue repair factors, growth and regulatory factors such as, but not limited to, oncostatine M, platelet-derived growth factors, fibroblast growth factors, epidermal growth factor, hepatocyte growth factor, bone morphogenic proteins, insulin-like growth factors, calcitonin and transforming growth factor alpha and beta. Preferably, the gene to be used in the invention encodes an interleukin receptor

confer enhanced IL-1ra activity on the expressed protein or they may confer enhanced stability, enhanced mucosal absorption or other properties on the expressed protein which render it even more suitable as a therapeutic agent for use in the methods of the invention. Thus, by "IL-1ra" as used herein is meant a protein having IL-1ra activity, which protein includes the wild type protein and mutants thereof having IL-1ra activity having characteristics substantially similar to wild type IL-1ra. Such properties include, for example, the ability of the mutated protein to bind IL-1 receptor and to act as an antagonist of IL-1 biological activity. Mutations of IL-1ra which retain IL-1ra activity include point mutations, deletions, insertions, frameshift mutations and other mutations which alter the primary sequence of the protein while preserving IL-1ra activity.

Preferably, the mutation in IL-1ra comprises an amino acid substitution at amino acid 91 (see Figure 4) wherein amino acid 91 is replaced by an amino acid selected from the group consisting of glutamine, arginine, lysine, histidine and tyrosine. The mutation may also comprise an amino acid substitution at position 109 wherein the amino acid at position 109 is replaced by an amino acid selected from the group consisting of serine, alanine, phenylalanine, valine, leucine, isoleucine and methionine. In some instances, the protein may be mutated such that there is an amino acid substitution in the protein at both positions 91 and 109. Preferably, in the mutated IL-1ra, amino acid 91 is replaced by arginine and/or IL-1ra amino acid 109 is replaced by alanine.

antagonist, and most preferably, the gene encodes IL-1 receptor antagonist (IL-1ra).

It is well known that proteins which are active only in glycosylated form must be expressed in microorganisms such as yeast.

It is however preferred the use of genes encoding eukariotic proteins active in non-glycosylated form as that they can be expressed in bacteria such as *B. subtilis*, *E. coli* or *Lactobacillus* species. It is also preferred the use of proteins which do not require an activation from a pro-form or post-expression processing.

Particularly preferred proteins which may be administered via engineered microorganisms according to the invention are IL-1ra, interferons, IL-10, growth hormone, alpha 1-antitrypsin.

Preferably, the gene to be used in the invention encodes an interleukin receptor antagonist, and most preferably, the gene encodes IL-1 receptor antagonist (IL-1ra).

Importantly, IL-1ra does not have IL-1 biological activity; rather, IL-1ra binds to IL-1 cell receptors without activating the cell, thereby functioning as an antagonist of IL-1 activity.

The preferred embodiment of the invention, the use of IL-1ra expressed in bacteria as a therapeutic treatment, should not be construed to be limited to wild type IL-1ra protein. As described in the example herein, the invention includes other forms of IL-1ra having IL-1ra activity, which forms of IL-1ra typically comprise mutations in the wild type protein. Such mutations may

Other amino acid substitutions are also contemplated by the invention and include substitutions which comprise conservative amino acid sequence differences compared with the wild type protein at positions other than amino acids 91 and 109. For example, conservative amino acid changes may be made, which although they alter the primary sequence of the protein or peptide, do not normally alter its function. Conservative amino acid substitutions typically include substitutions within the following groups:

glycine, alanine;
valine, isoleucine, leucine;
aspartic acid, glutamic acid;
asparagine, glutamine;
serine, threonine;
lysine, arginine;
phenylalanine, tyrosine.

The procedures for introduction of mutations into a gene are well known in the art and are described for example, in Sambrook et al., (1989, Molecular Cloning; A Laboratory Manual, Cold Spring Harbor, NY).

The types of diseases in humans which are treatable by administration of the pharmaceutical compositions of the invention include, inflammatory bowel diseases including Crohn's disease and ulcerative colitis (treatable with IL-1ra or IL-10); autoimmune diseases, including but not limited to psoriasis, rheumatoid arthritis, lupus erythematosus (treatable with IL-1ra or IL-10); neurological disorders including, but not limited to Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (treatable with brain

devated neurotropic factor and ciliary neurotropic factor); cancer (treatable with IL-1, colony stimulating factors and interferon- γ); osteoporosis (treatable with transforming growth factor β); diabetes (treatable with insulin); cardiovascular disease (treatable with tissue plasminogen activator); atherosclerosis (treatable with cytokines and cytokine antagonists); hemophilia (treatable with clotting factors); degenerative liver disease (treatable with hepatocyte growth factor or interferon α); pulmonary diseases such as cystic fibrosis (treatable with alpha-1 antitrypsin); and, viral infections (treatable with any number of the above-mentioned compositions).

The invention also contemplates treatment of diseases in other animals including dogs, horses, cats and birds. Diseases in dogs include but are not limited to canine distemper (paramyxovirus), canine hepatitis (adenovirus Cav-1), kennel cough or laryngotracheitis (adenovirus Cav-2), infectious canine enteritis (coronavirus) and haemorrhagic enteritis (parvovirus). Diseases in cats include but are not limited to viral rhinotracheitis (herpesvirus), feline caliciviral disease (calicivirus), feline infectious peritonitis (parvovirus) and feline leukaemia (feline leukaemia virus). Other viral diseases in horses and birds are also contemplated as being treatable using the methods and compositions of the invention. To this purpose, the use of microorganisms expressing recombinant interferons will be particularly preferred.

The recombinant microorganisms of the invention are suspended in a pharmaceutical formulation for

administration to the human or animal having the disease to be treated. Such pharmaceutical formulations include live microorganisms and a medium suitable for administration. The recombinant microorganisms may be lyophilized in the presence of common excipients such as lactose, other sugars, alkaline and/or alkali earth stearate, carbonate and/or sulfate (for example, magnesium stearate, sodium carbonate and sodium sulfate), kaolin, silica, flavorants and aromas. Microorganisms so lyophilized may be prepared in the form of capsules, tablets, granulates and powders, each of which may be administered by the oral route. Alternatively, some recombinant bacteria, or even spores thereof, may be prepared as aqueous suspensions in suitable media, or lyophilized bacteria or spores may be suspended in a suitable medium just prior to use, such medium including the excipients referred to herein and other excipients such as glucose, glycine and sodium saccharinate. For oral administration, gastroresistant oral dosage forms may be formulated, which dosage forms may also include compounds providing controlled release of the microorganisms and thereby provide controlled release of the desired protein encoded therein. For example, the oral dosage form (including tablets, pellets, granulates, powders) may be coated with a thin layer of excipient (usually polymers, cellulosic derivatives and/or lipophilic materials) that resists dissolution or disruption in the stomach, but not in the intestine, thereby allowing transit through the stomach in favour of disintegration, dissolution and absorption in the intestine. The oral dosage form may be designed

to allow slow release of the microorganism and of the recombinant protein thereof, for instance as controlled release, sustained release, prolonged release, sustained action tablets or capsules. These dosage forms usually contain conventional and well known excipients, such as lipophilic, polymeric, cellulosic, insoluble, swellable excipients. Controlled release formulations may also be used for any other delivery sites including intestinal, colon, bioadhesion or sublingual delivery (i.e., dental mucosal delivery) and bronchial delivery. When the compositions of the invention are to be administered rectally or vaginally, pharmaceutical formulations may include suppositories and creams. In this instance, the microorganisms are suspended in a mixture of common excipients also including lipids. Each of the aforementioned formulations are well known in the art and are described, for example, in the following references: Hansel et al. (1990, Pharmaceutical dosage forms and drug delivery systems, 5th edition, William and Wilkins); Chien 1992, Novel drug delivery system, 2nd edition, M. Dekker); Prescott et al. (1989, Novel drug delivery, J. Wiley & Sons); Cazzaniga et al, (1994, Oral delayed release system for colonic specific delivery, Int. J. Pharm. 108:77).

Thus, according to the invention, recombinant microorganisms encoding a desired gene may be administered to the animal or human via either an oral, rectal, vaginal or bronchial route. Dosages of microorganisms for administration will vary depending upon any number of factors including the type of bacteria and the gene encoded thereby, the type and

severity of the disease to be treated and the route of administration to be used. Thus, precise dosages cannot be defined for each and every embodiment of the invention, but will be readily apparent to those skilled in the art once armed with the present invention. The dosage could be anyhow determined on a case by case way by measuring the serum level concentrations of the recombinant protein after administration of predetermined numbers of cells, using well known methods, such as those known as ELISA or Biacore (See examples). The analysis of the kinetic profile and half life of the delivered recombinant protein provides sufficient information to allow the determination of an effective dosage range for the transformed microorganisms. As an example, *Bacillus subtilis* encoding IL-1ra may be administered to an animal at a dose of approximately 10^9 colony forming units (cfu)/kg body weight/day.

In a preferred embodiment of the invention, the recombinant gene is IL-1ra and the bacterium is *Bacillus subtilis*. IL-1ra is a protein which is structurally similar to that of IL-1 which binds with high affinity to IL-1 receptor but which does not activate target cells (Dinarello et al., 1991, Immunol. Today 11:404). IL-1ra therefore functions as an antagonist to the effects of IL-1 and has potential as a therapeutic agent useful for treatment of inflammatory and matrix-destruction diseases which are mediated through the action of IL-1. Such diseases include rheumatoid arthritis, osteoporosis and septic shock. However, in order to be useful in vivo as a therapeutic agent, it is

necessary in some cases that IL-1ra be given continuously in high doses.

Thus, according to the present invention, *Bacillus subtilis* which has been manipulated to encode IL-1ra is administered in vivo to an animal at various anatomical sites such as the peritoneal cavity, the small intestine or the large intestine. Recombinant IL-1ra, produced at the site of administration of the bacterium then functions in many cases, as a continuously produced therapeutic agent capable of counteracting the effects of IL-1 in that region of the animal.

While the examples provided include the nucleotide sequences for a variety of promoter regions and the coding sequence of human IL-1ra, the invention should also be construed to include other sequences which share substantial homology with those sequences presented herein.

"Homologous", as used herein, refers to the subunit sequence similarity between two polymeric molecules, e.g., between two nucleic acid molecules, DNA molecules or two RNA molecules, or between two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then they are homologous at that position. The homology between two sequences is a direct function of the number of matching or homologous positions, e.g., if half (e.g., five positions in a polymer ten subunits in length) of the positions in two compound sequences are homologous then the two sequences are 50% homologous, if 90% of the positions, e.g., 9 of

10, are matched or homologous, the two sequences share 90% homology. By way of example, the DNA sequences 3'ATTGCC5' and 3'TATGGC share 50% homology. By "substantial homology" as used herein refers to a nucleotide or amino acid sequence which is at least 50% homologous, preferably 60% homologous, more preferably 80% homologous and most preferably 90% homologous to a nucleotide or amino acid sequence described herein.

The following provides some examples of the present invention. These examples are not to be considered as limiting the scope of the appended claims.

EXAMPLES

Example 1. Examples of systems for expression of heterologous genes in bacteria

Bacillus subtilis. Constitutive expression of heterologous genes into Bacillus subtilis may be accomplished by cloning a cDNA encoding the protein to be expressed downstream of the bacteriophage T5 promoter region, comprising the sequence (Sequence Id N. 3):

5' TCTAGAAAAATTTATTTGCTTTCAGGAAAATTTTTATGTATAATAGATT
 XbaI -35 -10
CATAAATTTGAGAGCTCAAAGGAGGAATTCGAGCTCGGTACCCGGGGATCCTCT
 EcoRI SacI KpnI SmaI BamHI
AGAGTCGACCTGCAGGCATGCAAGCTT

XbaI SalI PstI SphI HindIII

The T5 promoter is cloned into a suitable vector such as pSM214 (Velati Bellini et al., 1991, Biotechnol. 18:177); a suitable strain of B. subtilis is for example, SMS118. The cDNA encoding the desired protein is cloned into the polylinker region shown above.

Cloning of heterologous genes in B. subtilis is

well known and is described, for example, in Sonenshein et al. (1993, *Bacillus subtilis* and Other Gram Positive Bacteria, American Society for Microbiology, Washington, D.C.).

5 Inducible expression of a heterologous gene in *Bacillus subtilis* is accomplished by cloning a cDNA encoding the desired protein downstream from a regulatory region comprising the T5 promoter region and the lac operator region. Inducible expression of the
10 desired protein is effected by IPTG-mediated inhibition of the LacI repressor gene expressed in an appropriate host strain (Schon et al., 1994, *Gene* 147:91-94). In this instance, a minimal T5 promoter region may be used comprising approximately the following nucleotide
15 (Sequence Id N. 4):

5'TTGCTTTCAGGAAAATTTTTTATGTATAATAGATTCATAAA

-35

-10

The lac operator region is cloned upstream and the heterologous gene is cloned downstream from this
20 sequence. The procedures to accomplish cloning are well known to those skilled in the art and are described, for example, in Sambrook et al. (1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY). Procedures for induction of gene expression via the lac operator or
25 other inducible expression systems are also well known in the art and are described in Sambrook (*supra*).

Escherichia coli. Constitutive expression of a heterologous protein in *Escherichia coli* may be accomplished in the same manner as described for
30 *Bacillus subtilis*. Additional cloning vectors and expression systems are described in Sambrook (1989,

Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY).

Inducible expression in *Escherichia coli* is accomplished using any number of procedures including that which is now described. A cDNA encoding the desired heterologous protein is cloned downstream of the promoter region of gene 10 of the bacteriophage T7 (Studier et al., 1986, J. Mol. Biol. 189:113-130) "and the cDNA coding for the T7-specific RNA polymerase is cloned under the control of lac promoter in the bacterial host strain such as *Escherichia coli* BL21 (DE3)". Inducible expression of the desired protein is effected by IPTG-mediated inhibition of the LacI repressor gene as described above.

The DNA sequence of the T7 promoter is as follows (Sequence Id N. 5) 5'TAATACGACTCACTATAGGGAGA. This region may be cloned into a suitable vector, such as the ones of the bluescript series (Stratagene, La Jolla, CA), expression vectors that use the T7 system (pRSET series Invitrogen Corporation, San Diego, CA or pET series Novagen Inc., Madison, WI). Additional elements may be added to this sequence (and to that described above for expression of genes in *Bacillus subtilis*) which provide a ribosome binding site (RBS), and ATG translation start codon and a multiple cloning region as follows (Sequence Id N. 6):

5'CGAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAA

T7 promoter

TAATTTTGTTTAACTTTAAGAAGGAGATATACATATG

RBS

NdeI

The NdeI site is the site of choice for cloning of

a cDNA encoding a heterologous protein.

Lactococcus lactis. Constitutive expression of heterologous genes cloned into Lactococcus lactis is accomplished using the p32 promoter which controls
5 expression of Lactococcus lactis fructose-1,6-diphosphate aldolase (Van de Guchte et al., 1990, Appl. Environ. Microbiol. 56:2606-2611). This region may be cloned into a suitable vector, for instance those derived essentially from pWV01 or from pSH71 (Kok et
10 al., 1984, Appl. Environ. Microbiol. 48:726). Another suitable expression vector is pMG36e (Van de Guchte, supra) and additional sequences may be added as described above which sequences include a ribosome binding site and a translation start codon.

15 Inducible expression of heterologous genes in Lactococcus lactis is accomplished using the T7 gene 10 promoter as described above (Wells et al, 1993, Mol. Microbiol. 8:1155-1162).

Lactobacillus spp. Constitutive expression of genes
20 in Lactobacillus spp. is accomplished using the Lactobacillus casei L(+)-lactate dehydrogenase promoter (Pouwels et al., 1993, Genetics of lactobacilli: plasmids and gene expression, Antonie van Leeuwenhoek 64:85-107). This promoter has the following sequence
25 (Sequence Id N. 7)

5'AAAAGTCTGTCAATTTTGTTCGGCGAATTGATAATGTGTTATACTCACAA

-35

-10

As described above, additional sequence elements may be added including a ribosome binding site and a
30 translation start codon.

A cDNA encoding the desired heterologous gene is

27

cloned downstream of this promoter region into a suitable vector, such as those disclosed in Pouwels et al., 1993, supra and in Posno et al. 1991, Appl. Environ. Microbiol. 57:1882.

5 Inducible expression of a heterologous gene in *Lactobacillus* spp. is accomplished using the alpha amylase promoter sequence of *Lactobacillus amylovirus*. This promoter is induced in the presence of cellobiose and is repressed in the presence of glucose (Pouwels et al., 1993, Genetics of lactobacilli; plasmids and gene expression, Antonie van Leeuwenhoek 64:85-107). The nucleotide sequence of the alpha amylase gene is as follows (Sequence Id N. 8)

10 5'GCAAAAAAATTTTCGATTTTATGAAAACGGTTGCAAAGAAGTTAGCAAAAA

15 glucose operator/-35

TATATAAT 3'

-10

TTCTTTTGAAATTGTTCACTTGGCCAAGCTGCAGTTTCAAATATTTTAAT
AAAGGGGGCAGTAAAAA.

20 RBS

This sequence is cloned into a suitable vector, and as described above, additional elements may be added including a translation start codon.

Inducible expression may also be accomplished using
25 the D xylose isomerase promoter region of *Lactobacillus pentosus*. This promoter is induced in the presence of xylose (Pouwels et al., 1993, Genetics of lactobacilli: plasmids and gene expression, Antonie van Leeuwenhoek 64:85-107). The nucleotide sequence of the D-xylose
30 isomerase promoter region is as follows (Sequence Id N. 9)

28

5'AGAAAGCGTTTACAAAAATAAGCCAATGCCGCTGTAATCTTAC 3'

-35

-10

As described herein, additional elements may also be added to this sequence including a ribosome binding site and a translation start codon.

Introduction of recombinant protein expressed by colonizing bacteria can be obtained by providing locally the appropriate inducer, for example by giving cellobiose orally for the amy-driven expression system.

Example 2. Mutant and wild type forms of IL-1ra having IL-1ra activity

Cloning and expression of human recombinant IL-1ra in Escherichia coli was performed as follows. A cDNA encoding IL-1ra was obtained by performing RT-PCR on a cDNA pool of RNAs expressed in monocyte/macrophage cells using standard methods (Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY). The primers used for amplification were as follows (Sequence Id N. 10 and 11)

IL-1ra forward - 5' GATCATATGCGACCTCTGGGAGAAAATCC 3'

NdeI Arg

IL-1ra reverse - 5' GATCTGCAGCTACTCGTCCTCCTGGAAG 3'

PstI

The forward primer was designed to create an NdeI site having an ATG translation start codon, the NdeI site being immediately upstream from the first amino acid of the mature IL-1ra protein (Arginine, R26). The reverse primer was designed such that it contains a PstI site downstream from the translation stop codon in the IL-1ra cDNA. The amplified fragment was cloned into the NdeI and PstI sites of the plasmid, pRSETA-IL-1ra. This

plasmid was introduced into *Escherichia coli* strain BL21 (DE3) (F^- , ompT, hsdD_B, ($r_B^-m_B^-$) gal, dom, (DE3)) using standard procedures. A map of the expression plasmid pSETA-IL-1ra is shown in Figure 1.

5 To obtain mutations in IL-1ra, a portion of the coding region of the IL-1ra gene (that encoding amino acids 30-152) is excised from the plasmid pRSETA-IL-1ra and is recloned into the mutagenesis Bluescript plasmid SK⁺ between the PstI sites thereby generating the
10 plasmid BSK-IL-1ra. Mutagenesis of the gene is effected using synthetic oligonucleotides obtained using an Applied Biosystems 392 oligonucleotide synthesizer and phosphoramidite chemistry.

To mutate the IL-1ra gene at the codon coding for
15 amino acid asparagine 91 the complementary sequence (Sequence Id N. 12)

5' GCT CTG CTT TCT GCG CTC GCT CAG 3'

91

was used. To obtain the plasmid BSK-MILRA-1 (containing
20 a mutation at codon 91) the synthetic oligonucleotide was mixed with single stranded plasmid BSK-IL-1ra DNA in a standard hybridization buffer (containing 5 pmol of oligonucleotide and 0,2 pmol of single stranded DNA in 10 ml of buffer). The mixture was heated to 70°C, it was
25 cooled slowly to 30°C for 40 minutes and was placed on ice. One Ml of standard synthesis buffer, 1 Ml (3 units) of T4 DNA ligase and 1 Ml (0,5 units) of T7 DNA polymerase were added to the mixture. Following 1 hour of incubation at 37°C, the mixture was used to transform
30 competent cells. Identification of positive clones is performed by nucleotide sequence analysis.

Mutated IL-1ra comprising an amino acid substitution at position 109 was generated in a similar manner using the synthetic oligonucleotide

5' CTC AAA ACT GGC CGT GGG GCC 3' (Sequence id N. 13)

5 In each instance, a different codon may be used to generate a mutated IL-1ra having different amino acid substitutions at either or both of positions 91 or 109 using the procedures and oligonucleotides described above by inserting the desired codon at the appropriate position.

Each of the mutated sequences is then cloned into an expression vector, for example, the sequences may be cloned back into the vector pRSETA-IL-1ra between sites SpeI and PstI generating a variety of expression plasmids comprising mutated IL-1ra. The plasmid comprises a mutation in amino acid position 91 of IL-1ra cloned into the vector RSETA-IL-1ra. This plasmid (T7MILRA-1) encodes an arginine residue at position 91 and expresses mutated IL-1ra having biological activity of IL-1ra (see below). Similarly, plasmid pT7MILRA-2 encodes mutated IL-1ra comprising the amino acid substitution Ala in the place of Thr in position 109 and gives rise to plasmid BSK-MILRA-2, and plasmid pT7MILRA-3 encodes mutated IL-1ra comprising both amino acid substitutions Arg in the place of Asn in position 91 and Ala in the place of Thr in position 109 and gives rise to plasmid BSK-MILRA-3.

Example 3. Generation of *Bacillus subtilis* capable of producing large amounts of recombinant protein

30 The natural process of sporulation of *Bacillus*, which process induces natural lysis of vegetative cells,

is exploited in order that large amounts of heterologous protein may be expressed in and recovered from this organism. Although the examples given herein relate to the genus *Bacillus*, the invention should be construed to encompass other sporulating bacteria, such as those in the genus *Clostridium*.

Sporulation of *Bacillus* is induced by placing the bacteria in medium suitable for sporulation (Jongman et al., 1983, Proc. Natl. Acad. Sci. USA 80:2305). Both vegetative growth and sporulation may be obtained in the same medium, i.e., potato extract medium, provided the cell concentration is high (Johnson et al., 1981, J. Bacteriol. 146:972). During sporulation, the bacterial vegetative cells lyse; thus, following sporulation, expressed proteins are released from the lysed cells and recovered in the supernatant.

Thus, we have evidence that sporulating bacteria effectively produce recombinant proteins in the sporulation supernatant.

Moreover, the following data provide guide-lines for the applicability of the invention.

The experiments described below exemplify (i) cloning, expression and release of a recombinant protein, IL-1RA, in *Bacillus*; (ii) purification of a recombinant protein so expressed;

(i) Cloning, expression and release of recombinant IL-1ra from *Bacillus*. A cDNA encoding IL-1ra was obtained using a reverse transcriptase (RT) polymerase chain reaction (PCR) from a cDNA library of RNAs transcribed in monocyte/macrophage cells using standard methods (Sambrook et al., 1989, Molecular Cloning: A

Laboratory Manual, Cold Spring Harbor, NY). The primers used for amplification were as follows (Sequence Id N. 14 and 15)

IL-1ra forward - 5'GGGAATTCTTTATGCGACCCTCTGGGAGAAAATCC 3'

5 EcoR I

IL-1ra reverse - 5'GGCTGCAGCTACTCGTCCTCCTGGAAG 3'

PstI

The forward primer was designed to create an EcoRI site just 5' to the initial ATG codon upstream of the first codon of the mature protein (R26). Similarly, the reverse oligonucleotide was designed to introduce a PstI site downstream of the translation stop codon of the protein. The amplified fragment was cloned into the EcoRI PstI sites in the vector pSM214 thereby generating pSM441 (Figure 2). This plasmid was used to transform *Bacillus subtilis* strain SMS 18 (leu⁻, pyrDL, npr⁻, apr⁻) using standard techniques. The transformed culture was maintained at -80°C in glycerol until use.

Generation of mutated IL-1ra proteins in *Bacillus subtilis* is performed by cloning the SpeI-PstI restriction fragments of BSK-MILRA-1, BSK-MILRA-2 or BSK-MILRA-3 in pSM441 between the same sites.

To generate IL-1ra in *Bacillus subtilis* which expressed extracellularly, the forward primer which is used contains the forward primer sequence provided above, in addition to the sequence encoding the protein subtilis in which is essential for the secretion of the protein from cells. The nucleotide sequence of this forward primer is as follows (Sequence Id N. 16)

30 5'GGGAATTCTTTATGAGAAGCAAAAAATTGTGGATCAGCTTGTTGTTTGCGTTAA
CGTTAATCTTTACGATGGCATTGAGCAACATGAGACGCGTCCGGCGACCCTCTGGG

AGAAAATCC 3'

The reverse primer in this reaction is as described above. An amplified fragment comprising these sequences is cloned into the corresponding EcoRI and PstI sites in the expression vector pSM241 to generate the plasmid pSM441sec. Transformation of *Bacillus subtilis* with this plasmid is as described above for the plasmid pSM441.

To generate IL-1ra, two aliquots of 100 ml of Luria broth (LB) medium containing 5 mg/l of chloramphenicol were inoculated with a 1:200 dilution (in LB) of primary inoculum derived from the glycerol stock. The cultures were incubated for 7 hours at 37°C with shaking. Bacteria were harvested by centrifugation at 3000 x g for 20 minutes at 4°C. The cells were washed once in 50 mM Morpholinoethansulfonic acid (MES)-NaOH solution, pH 6.25 containing 1 mM EDTA (Buffer A). At this point, cells were either stored at 80°C until preparation of lysates, or they were processed immediately for sporulation.

To prepare lysates, the cells were thawed and were resuspended in 10 ml of Buffer A and were then disrupted using an XL2020 sonicator (Heat System, Farmingdale, NY) at an output power of 80W for 14 cycles of 30 seconds of sonication and 30 seconds in ice water. The cell lysate was centrifuged at 30,000 x g for 30 minutes at 4°C and the supernatant was recovered while avoiding the layer of lipid at the top of the centrifuge tube. The lysate was stored at -80°C until use. This supernatant was termed "Sc".

To induce sporulation, bacteria were resuspended in 10 ml of Difco sporulation medium without

chloramphenicol (3 g/l Bacto beef extract, 5 g/l Peptone, 0.25 mM NaOH, 10 mM MgSO_4 , 0.1% KCl, 0.1 mM MnCl_2 , 1 mM $\text{Ca}(\text{NO}_3)_2$ and 1 mM FeSO_4 at pH 6.8). Cells were incubated for approximately 12 hours at 35°C with shaking. Spores which had formed were harvested by centrifugation at 3,000 x g for 20 minutes at 4°C. The supernatant was stored at -80°C until use. This supernatant was termed "Ss".

(ii) Purification and assessment of IL-1ra. To obtain IL-1ra, supernatants Sc and Ss were thawed and filtered through a Millex HV 0.45 mm unit (Millipore). The pH of supernatant Ss was adjusted to 6.25 using HCL. Both supernatants were incubated for 3 hours at 4°C with gentle shaking in the presence of 1 ml of Q-Sepharose Fast Flow anionic exchanger (Pharmacia). The anionic exchanger was equilibrated in Buffer A prior to incubation with the supernatants and a column comprising the same wax formed in a Poly-Prep disposable column (Bio-Rad). Non-adsorbed material was collected and incubated as described above in the presence of 1 ml of S-Sepharose Fast Flow cationic exchanger (Pharmacia) which had been equilibrated in Buffer A. After washing in Buffer A, the S-Sepharose was batch eluted using 2 ml of Buffer A containing 0.5 M NaCl. The eluted material was concentrated to a volume of 0.25 ml using a Centricon 10 centrifugal concentrator (Amicon).

Proteins which were obtained as described above were analyzed by electrophoresis through 13.5% mini SDS-PAGE and were visualized by staining using standard technology. The protein bands were further analyzed by laser scanning using a Molecular Dynamics Personal

Densitometer and a densitometric image was obtained using Image Quant software (Molecular Dynamics). The results of this analysis are summarized in Figure 3 and in Table 1. It is apparent from these results that IL-1ra was expressed in these cells.

TABLE 1

Determination of IL-1ra in the cell lysate supernatant (Sc) and in the Sporulation supernatant (Ss) of *Bacillus subtilis* engineered to express human IL-1ra intracellularly

Sample	Total Protein (mg)	IL-1ra ⁽¹⁾		Biological Activity ⁽²⁾
		%	mg	
Sc: crude	12.5	10	1.250	n.t.
enriched	0.675	66	0.445	0.9×10^6
Ss: crude	9.6	12	1.152	n.t.
enriched	0.440	80	0.350	1.1×10^6

(1) Determined by laser scanning densitometry of SDS-PAGE.

(2) Specific activity is expressed in inhibitory units (IU)/mg IL-1ra. Specific activity of an IL-1ra standard preparation (human recombinant IL-1ra from *Escherichia coli*) is 0.9×10^6 IU/mg.

n.t. Not tested.

The amount of protein in each of the above-described preparations was assessed using a standard Bio-Rad protein assay following the manufacturer's instructions. Bio-Rad protein standard I was used as the standard when assessing the amount of

protein in the starting material. A purified standard of recombinant IL-1ra was used as a standard to assess the amount of IL-1ra in the purified fractions. The concentration of IL-1ra was determined by measuring the absorption at 280 nm (A_{280}) of the protein in a 6 M guanidinium hydrochloride solution in 20 mM phosphate buffer, pH 6.5. Under these conditions, the $A_{280}^{0.1\%}$ of IL-1ra calculated using PC/GENE software (Intelligenetics) was 0.910.

10 The biological activity of IL-1ra was measured in a murine thymocyte proliferation assay. Thymocytes plated at a concentration of 6×10^5 cells per well of a 96 well plate were obtained from 5 to 7 week old C3H/HeJ mice (The Jackson Laboratories). The thymocytes were

15 cultured in RPMI-1640 (Gibco) and 5% heat-inactivated fetal bovine serum (Hyclone), 25 mM betamercaptoethanol, 50 mg/ml gentamicin (Sigma) and 1.5 mg/ml PHA (Wellcome). A fixed concentration of recombinant human IL-1 beta (1 unit = 30 pg/ml) either alone or in the

20 presence of differing amounts of standard IL-1ra or IL-1ra containing fractions (at concentrations of 10 pg/ml to 100 ng/ml) was added to triplicate wells. Following addition, thymocytes were incubated at 37°C in moist air having 5% CO_2 for 72 hours. An amount of 0.5mCi of

25 ^3H -thymidine (DuPont-NEN) was added and incubation was continued overnight. Cells were then harvested onto glass fiber filters. The level of incorporation of ^3H -thymidine into the cells was determined by scintillation counting. One unit of IL-1ra is calculated

30 as the amount which inhibits 50% of IL-1 induced proliferation. The data which are provided in Table 1

supernatant were analyzed by electrophoresis and Western blotting. Rabbit anti-IL 1ra antibody was diluted 1:100,000 and was conjugated to GAR/BRP (Bio-Rad) at a 1:15,000 ratio and an IBI Enzygraphic Web (Kodak) system was used to detect the presence of the proteins. As a positive control, experiments were conducted wherein 10 ng of IL-1ra was added to a sample of ra-negative serum. The results which are presented in Table 2 reflect the relative amounts of IL-1ra which were detectable in each sample using this method. It is evident that IL-1ra is detectable in the mice, in particular, by 3 hours post administration. Anti-IL-1ra antibody was prepared in rabbits which were inoculated with recombinant IL-1ra using standard procedures (Harlow et al., 1988, In: Antibodies, A Laboratory Manual, Cold Spring Harbor, NY). As a positive control, experiments were conducted wherein 10 ng of IL-1ra was added to a sample of IL-1ra-negative serum. The results which are presented in Table 2 reflect the relative amount of IL-1ra which were detected in each sample using this method. It is evident that IL-1ra may be detected in the mice, in particular, by 3 hours post administration, following administration of bacteria expressing the same.

suggests that there are no significant differences between the activity of IL-1ra in preparations of lysates or preparations of sporulating bacteria of the invention.

5 Example 4. Expression of IL-1ra in *Bacillus subtilis* in vivo

 The nucleotide and corresponding amino acid sequence of human IL-1ra is given in Figure 4. *Bacillus subtilis* encoding IL-1ra was administered
10 intraperitoneally to mice. At various times post-administration, which times are indicated in the accompanying Table 2, peritoneal samples were obtained by lavage of the peritoneum and in addition, serum samples were obtained. Western blot analysis was
15 performed on each sample using a rabbit anti-recombinant human IL-1ra antibody.

 Essentially, *Bacillus subtilis* was propagated in LB medium in the presence of 5 mg/l of chloramphenicol at 37°C overnight with continuous stirring. The bacteria
20 were collected by centrifugation and the cells were resuspended in phosphate buffered saline (PBS). Female C3H/HeOuJ mice, having a body weight of approximately 20 g, were inoculated with 3×10^7 bacteria per mouse in 0.2 ml PBS. Untreated animals served as controls.

25 At 3, 6 and 24 hours post-administration, peritoneal lavage was performed using 3 ml of physiological saline. Two animals were sacrificed at each time point. Serum samples were also obtained from the mice at the indicated times. The lavage samples were
30 centrifuged at 5,000 x g for 30 minutes at 4°C and the supernatant was recovered. Aliquots of 1 and 3 µl of

TABLE 2

Identification of IL-1ra BY Western blot analysys after in vivo administration of engineered Bacillus subtilis.

5	SAMPLE		
	SAMPLING TIME		
	Peritoneal lavage		serum
10	Basal	-	-
	3 hours	+++	+
15	6 hours	+	-
	24 hours	-	-

Example 5. Release of IL-1ra from Bacillus subtilis administered in vivo to rats

Male Sprague-Dawley rats weighing about 200-250 g were fasted for 24 to 48 hours. The rats were anaesthetized with phenobarbital (45 mg/kg administered intramuscularly) or with ether. The abdomen was opened along the linea alba to permit access to the gastrointestinal tract. The end portion of the colon and/or the end portion of the ileum (0.5-1 cm above the ileocaecal valve) were identified and isolated using a ligature.

For administration of bacteria into the large intestine, volumes of 5 to 10 ml of 0.25 X LB (i.e., LB having one quarter of the concentration of the components normally contained therein) containing 3 to 6 $\times 10^8$ bacteria (propagated as described herein) were

aspirated with a syringe and were injected above the ileocaecal valve such that the caecum and a large portion of the colon was filled. An occlusion was formed by surgical ligature immediately below to point of entry of the needle so as to prevent bacteria from entering the peritoneal cavity.

For administration of bacteria to the small intestine, volumes ranging from 1 to 10 ml, containing 3 to 6×10^8 bacteria were injected into the proximal portion of the small intestine about 0.5 to 1 cm below the pylorus. As described above, an occlusion was formed by surgical ligature immediately below the point of entry of the needle to prevent entry of bacteria into the peritoneal cavity.

At 3, 4, 6, 8 and 24 hours post-administration, the animals were sacrificed and the abdomen was opened to expose the intestines. The intestinal lumen was washed with 10-50 ml of sterile physiological saline at constant pressure (100 cm H₂O) by means of a needle introduced into the caecum or the duodenum depending upon the site of administration. Aliquots of lavage (1-2 ml) were centrifuged at 5,000 x g for 30 minutes at 4°C and the amount of IL-1ra contained therein was assessed by Western blotting as described. These results are presented in Table 3. It is evident from the data that IL-1ra was released in both the large and small intestine following administration of *Bacillus subtilis* encoding the same. A greater amount of IL-1ra was present in the large intestine compared with the small intestine and IL-1ra appeared to persist in the large intestine for a longer period of time.

TABLE 3

Identification of IL-1ra by Western blot analysis in the intestinal lumen following in situ administration of engineered *Bacillus subtilis*

TIME (hours)						
INTESTINAL TRACT						
	Basal	3	4	5	8	24
Small Intestines	-	++	++	+	-	-
Large Intestine	-	+++++	+++++	++	++	++

Example 6. Protection of mice from endotoxic shock following intraperitoneal administration of *Bacillus subtilis* encoding IL-1ra

Female C3H/HeOuJ mice having a body weight of approximately 25 g were administered a single dose of *Bacillus subtilis* intraperitoneally as described. The mice each received 3×10^6 bacteria in 0.2 ml PBS. Control mice included those which received wild type *Bacillus subtilis* and mice which received PBS alone. At 24 hours post-administration, all of the animals were administered 15-20 mg/kg of LPS by intraperitoneal injection. Each test group comprised 10-20 animals. The number of deaths in each group of mice was recorded until 5 to 7 days following administration of LPS when no further deaths occurred within a 48 hour period. These data are presented in Table 4. From these data it is evident that inoculation of mice with *Bacillus subtilis* encoding IL-1ra enabled these mice to survive

lethal doses of LPS.

TABLE 4

Inhibition of endotoxic shock in mice treated with engineered bacillus subtilis

MORTALITY AFTER TREATMENT WITH:			
DOSE OF LPS			
PBS		B. subtilis wt	B. subtilis-il-1ra
15 mg/kg	40%	40%	0%
20 mg/kg	100%	100%	70%

Example 7. Expression of IL-1ra in Bacillus subtilis administered to rabbits

The strains of Bacillus subtilis which were used in the experiments described below were as follows: Strain SMS118 containing the plasmid pSM539, encodes human IL-1ra which is expressed intracellularly; strain SMS118 containing the plasmid pSM261, encodes human mature IL-1 beta which is expressed intracellularly; strain SMS118 containing the plasmid pSM214 deriving from pSM671 encodes beta-lactamase which is expressed in secreted form: each of these strains has the genotype leu⁻, pyrDL, npr⁻, apr⁻ pSM261 and pSM214 are disclosed in Velati Bellini et al. 1991, J. Biotechnol. 18:177. pSM539 is disclosed in IT-A-MI94001916. Bacteria were propagated and lysates were prepared therefrom as described herein.

Female New Zealand rabbits having a body weight of 1.9 to 2.3 kg were maintained in standard cages at

22±1°C under a 12 hour light 12 hour dark cycle. The rabbits received 100 g of a standard diet daily and water ad libitum.

For administration of *Bacillus subtilis* to the colon of the rabbits, the rabbits were fasted for a period of 16 to 18 hour treatment. The rabbits were then placed in standard stock restraints and were acclimated for 1 hour prior to treatment. A rounded tip urethral catheter (Rush, Germany) was gently inserted into the distal colon via the anus. Approximately 10 cm of the catheter was inserted. A suspension of *Bacillus subtilis* in 2 ml of PBS was administered through the catheter while the rabbits remained conscious.

To determine the temperature of the rabbits, rabbits were gently restrained in conventional stocks throughout the experiment and in each case, prior to experimentation, were acclimated to the stocks for about 2 hours. The body temperature of the animals was measured by means of a cutaneous thermistor probe (TM-54/S and TMN/S; L.S.I. Italy) which was positioned between the left posterior foot and the abdomen and was allowed to stabilize for 2 minutes. A suspension of *Bacillus subtilis* (containing plasmids pSM214 or pSM539 at 2×10^9 cells per rabbit) was instilled in the distal colon 1 hour before intravenous administration of highly purified LPS-free recombinant human IL-1 beta in pyrogen-free saline through the marginal ear vein. The temperature of the animals was recorded every 20 minutes for 3 hours beginning at the time of administration of IL-1 beta. To measure IL-1 induced hypoferremia in the rabbits, the concentration of iron in the rabbits was

assessed in samples obtained at 2, 4, 6, 8 and 24 hours following intravenous inoculation of 1 microgram/kg of recombinant human IL-1 beta. The iron assay was a colorimetric assay using a commercially available kit (Fe; Boehringer Mannheim, Mannheim, Germany). Normal iron levels prior to administration of IL-1 beta was 134.7 ± 6.6 micrograms/dl (mean \pm of 25 animals). Hypoferremia (a 60-75% decrease in the level of iron in the plasma) was evident at about 4 to about 24 hours following IL-1 beta administration. *Bacillus subtilis* strains containing pSM214 or pSM539 at a concentration of 2×10^9 cells per rabbit were instilled into the distal colon twice, once at 3 hours prior to administration of IL-1 beta and once at 10 minutes after IL-1 beta administration.

To detect IL-1ra in rabbit serum, serum was obtained from the animals through the marginal ear vein at 0, 1, 2, 4 and 8 hours following administration of *Bacillus subtilis*. Quantitative determination of IL-1ra was accomplished in a specific ELISA (Amersham International, Amersham, UK) following the manufacturer's instructions. In this assay, the lower detection limit is 20 pg/ml. Purified IL-1ra was used as a standard.

IL-1ra in serum was also measured using a Biosensor BIAcoreTM system (Pharmacia Biosensor AB, Uppsala, Sweden) which allows real time biospecific interaction analysis by means of the optical phenomenon of surface plasmon resonance (Lofas et al., 1990, J. Chem. Soc. Chem. Commun. 21:1526). The immobilization run was performed at a flow of 5 μ l/minute in HBS at pH 7.4 (10

mm HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.05% Surfactan P20 as described (Lofas et al., 1990, J. Chem. Soc. Chem. Commun. 21:1526; Fagerstam et al., 1990, J. Mol. Recogn. 3:208). Purified polyclonal IgG anti-IL-1ra was linked
5 via primary amino groups to the dextran matrix of a CM5 sensor chip according to the following optimized procedure. The carboxylated matrix of the sensor chip was first activated with 45 μ l of a 1:1 mixture of N-hydroxysuccinimide and N-ethyl-N'-(3 diethylamino-propyl-) carbodiimide. Then 70 μ l of antibody solution
10 (150 μ g/ml in 10 ml sodium acetate at pH 4.5) was injected. The free amino groups were blocked with 40 μ l of 1 M ethanolamine hydrochloride. Samples containing IL-1ra were introduced in a continuous flow passing over
15 the surface of the sensor chip, allowing interaction with the immobilized antibodies. Interaction was detected in terms of resonance angles and was expressed in resonance units (RU). For evaluation of serum IL-1ra concentration, a standard curve of IL-1ra in HBS was
20 constructed (from 0.1 ng/ml to 1 μ g/ml at a flow rate of 3 μ l/minute). Rabbit serum samples were filtered on Centricon Plus (Amicon, Beverly, MA) before analysis. The concentration of IL-1ra in serum samples was calculated by comparing the sample RU to the standard
25 curve using a Pfit program.

The results of the experiments now described are presented as the mean \pm SEM. Body temperature differences were assessed by one-way ANOVA and the significance was designed at the 95% confidence level.

30 Induction of in vitro sporulation in *Bacillus subtilis* manipulated to express IL-1ra intracellularly

(Figure 5A) renders this bacterium capable of releasing large amounts of intact, active recombinant IL-1ra (Figure 5B) within a few hours following induction of sporulation (Figure 5C). IL-1ra was also detectable in the serum of rabbits administered intracolonicallly with bacteria capable of producing IL-1ra (pSM539) but not with control beta-lactamase expressing bacteria (pSM214). When rabbits were administered intracolonicallly bacteria capable of producing IL-1ra (pSM539) or control beta-lactamase (pSM214) IL-1ra was also observed to be expressed (Figure 6). Here, the presence of IL-1ra, as measured by a specific ELISA, was observed for several hours in the rabbit serum following a single inoculation of 2×10^9 live *Bacillus subtilis* comprising pSM539. The rabbits which were administered pSM214 tested negative in the ELISA. The presence of IL-1ra in rabbit serum following treatment was confirmed in the BIAcore assay (Figure 7).

The ability of IL-1ra delivered by *Bacillus subtilis* to antagonise the effects of parenterally administered IL-1 beta was investigated. In Figure 8 it can be seen that the increase in body temperature induced in rabbits following intravenous injection of IL-1 beta was significantly reduced in rabbits which had received pSM539 compared with rabbits which had received pSM214. Further, this reduction was dose dependent. In addition, IL-1 beta induced hypoferrremia was reversed in rabbits treated with pSM539 compared with rabbits treated with pSM214.

To further establish that administration of bacteria to animals is an effective drug delivery

method, rabbits were administered *Bacillus subtilis* comprising pSM261 (encoding IL-1 beta). Rabbits which were administered this type of bacterium exhibited a significant increase in body temperature, up to 40 of
5 the animals died and, generally, the rabbits presented as if they had been administered IL-1 beta intravenously (Figure 9).

Example 8. Expression of IL-1ra in *Escherichia coli* administered to mice

10 To express IL-1ra in this *Escherichia coli*, the *Escherichia coli* transformed with the plasmid, pT7MILRA-3, were grown in LB containing 100 mg/l ampicillin until an optical density of 0.4 to 0.7 at 600 nm was reached.

15 Expression of the protein was then induced by the addition of a final concentration of 2-4 mM of IPTG to the medium. The cells were incubated for 2.5-3.0 hours at 37°C with shaking. Bacteria were then collected by centrifugation and were resuspended in a suitable volume of saline for administration to mice.

20 Female C3H/HeJ mice having a body weight of approximately 20-25 g were administered a single subcutaneous dose of *Escherichia coli* comprising the plasmid pT7MILRA-3. The number of bacteria administered ranged from 5×10^7 to 1.5×10^{10} cells/dose; the time
25 of administration of *Escherichia coli* to the mice varied from 1 to 3 hours prior to administration of IL-1 β to the mice. In all cases, the results obtained, i.e., the efficacies of the IL-1ra in the mice were comparable.

30 At time zero, mice were administered 4 μ g/kg of recombinant human IL-1 β intraperitoneally. Two hours post-administration of IL-1 β , the mice were sacrificed

and samples of serum were obtained. The level of glucose in the serum was assessed by reaction with glucose-oxidase using a commercially available kit (Glucose GOD Perid: Boehringer Mannheim). The results are presented in Figure 10. The extent of hypoglycemia induced by intraperitoneal IL-1 β is shown (filled column). Hypoglycemia was markedly reduced in mice pretreated with IL-1ra expressing *Escherichia coli* (hatched column). As a positive control, purified IL-1ra (4 mg/kg) was administered subcutaneously 15 minutes before administration of IL-1 β and was observed by IL-1 β (cross-hatched column). As a negative control, mice were subcutaneously administered *Escherichia coli* strain BL21 (D3) containing the plasmid pRSET-A (i.e., a plasmid which does not encode IL-1ra) prior to administration of IL-1 β . IL-1 β induce hypoglycemia in these mice was unaffected by administration of *Escherichia coli* containing pRSET-A establishing that it is the expression of IL-1ra in *Escherichia coli* which accounts for the observed reduction in hypoglycemia in the animals.

The data described herein thus establish that bacteria which express recombinant proteins when administered to an animal serve as efficient and effective drug delivery vehicles for treatment of disease in the animal.

The data described herein thus establish that bacteria which express recombinant proteins when administered to an animal serve as efficient and effective drug delivery vehicles for treatment of disease in the animal. Intracolonic administration of

bacteria establishes a source of drug for the animal having therapeutic effect.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Dompe' s.p.a.
- (B) STREET: via Campo di Pile
- (C) CITY: L'Aquila
- (E) COUNTRY: ITALY
- (F) POSTAL CODE (ZIP): I-67100
- (G) TELEPHONE: +39 862 3381
- (H) TELEFAX: +39 862 338219

(ii) TITLE OF INVENTION: Microorganisms as therapeutic delivery systems

(iii) NUMBER OF SEQUENCES: 16

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 531 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

51

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..531

(ix) FEATURE:

(A) NAME/KEY: sig_peptide

(B) LOCATION: 1..75

(ix) FEATURE:

(A) NAME/KEY: mat_peptide

(B) LOCATION: 76..531

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATG GAA ATC TGC AGA GGC CTC CGC AGT CAC CTA ATC ACT CTC CTC CTC	48
Met Glu Ile Cys Arg Gly Leu Arg Ser His Leu Ile Thr Leu Leu Leu	-10
-25	-15
TTC CTG TTC CAT TCA GAG ACG ATC TGC CGA CCC TCT GGG AGA AAA TCC	96
Phe Leu Phe His Ser Glu Thr Ile Cys Arg Pro Ser Gly Arg Lys Ser	5
-5	1
AGC AAG ATG CAA GCC TTC AGA ATC TGG GAT GTT AAC CAG AAG ACC TTC	144
Ser Lys Met Gln Ala Phe Arg Ile Trp Asp Val Asn Gln Lys Thr Phe	
10	15
	20

TAT CTG AGG AAC AAC CAA CTA GTT GCT GGA TAC TTG CAA GGA CCA AAT 192
 Tyr Leu Arg Asn Asn Gln Leu Val Ala Gly Tyr Leu Gln Gly Pro Asn 25 30 35

 GTC AAT TTA GAA GAA AAG ATA GAT GTG GTA CCC ATT GAG CCT CAT GCT 240
 Val Asn Leu Glu Glu Lys Ile Asp Val Val Pro Ile Glu Pro His Ala 40 45 50 55

 CTG TTC TTG GGA ATC CAT GGA GGG AAG ATG TGC CTG TCC TGT GTC AAG 288
 Leu Phe Leu Gly Ile His Gly Gly Lys Met Cys Leu Ser Cys Val Lys 60 65 70

 TCT GGT GAT GAG ACC AGA CTC CAG CTG GAG GCA GTT AAC ATC ACT GAC 336
 Ser Gly Asp Glu Thr Arg Leu Gln Leu Glu Ala Val Asn Ile Thr Asp 75 80 85

 CTG AGC GAG AAC AGA AAG CAG CAC AAG CCC TTC GCC TTC ATC CGC TCA 384
 Leu Ser Glu Asn Arg Lys Gln Asp Lys Arg Phe Ala Phe Ile Arg Ser 90 95 100

 GAC AGT GGC CCC ACC ACC AGT TTT GAG TCT GCC GGC TGC CCC CGT TGG 432
 Asp Ser Gly Pro Thr Thr Ser Phe Glu Ser Ala Ala Cys Pro Gly Trp 105 110 115

 TTC CTC TGC ACA GCG ATG GAA GCT GAC CAG CCC GTC AGC CTC ACC AAT 480
 Phe Leu Cys Thr Ala Met Glu Ala Asp Gln Pro Val Ser Leu Thr Asn 120 125 130 135

 ATG CCT GAC GAA GGC GTC ATG GTC ACC AAA TTC TAC TTC CAG GAG GAC 528
 Met Pro Asp Glu Gly Val Met Val Thr Lys Phe Tyr Phe Gln Glu Asp 140 145 150

 GAG 531
 Glu

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 177 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```

Met Glu Ile Cys Arg Gly Leu Arg Ser His Leu Ile Thr Leu Leu Leu
-25      -20      -15      -10
Phe Leu Phe His Ser Glu Thr Ile Cys Arg Pro Ser Gly Arg Lys Ser
      -5      1      5
Ser Lys Met Gln Ala Phe Arg Ile Trp Asp Val Asn Gln Lys Thr Phe
      10      15      20
Tyr Leu Arg Asn Asn Gln Leu Val Ala Gly Tyr Leu Gln Gly Pro Asn
      25      30      35
Val Asn Leu Glu Glu Lys Ile Asp Val Val Pro Ile Glu Pro His Ala
      40      45      50      55
Leu Phe Leu Gly Ile His Gly Gly Lys Met Cys Leu Ser Cys Val Lys
      60      65      70
Ser Gly Asp Glu Thr Arg Leu Gln Leu Glu Ala Val Asn Ile Thr Asp
      75      80      85
Leu Ser Glu Asn Arg Lys Gln Asp Lys Arg Phe Ala Phe Ile Arg Ser
      90      95      100

```

Asp Ser Gly Pro Thr Thr Ser Phe Glu Ser Ala Ala Cys Pro Gly Trp
 105 110 115
 Phe Leu Cys Thr Ala Met Glu Ala Asp Gln Pro Val Ser Leu Thr Asn
 120 125 130 135
 Met Pro Asp Glu Gly Val Met Val Thr Lys Phe Tyr Phe Gln Glu Asp
 140 145 150

Glu

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 131 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bacteriophage T5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TCTAGAAAAA TTTATTGCT TTCAGGAAA TTTTATATGT ATAATAGATT CATAAATTG	60
AGAGCTCAA GGAGGAATTC GAGCTCGGTA CCCGGGGATC CTCTAGAGTC GACCTGCAGG	120
CATGCAAGCT T	131

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bacteriophage T5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TTGCTTTCAG GAAATTTT TATGTATAAT ACATTCATAA A

41

55

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bacteriophage T7

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TAATACGACT CACTATAGGG AGA

23

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 89 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bacteriophage T7

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CGAAATTAAT ACCACTCACT ATAGGAGAC CACAACGGTT TCCCTCTAGA AATAATTTTG 60

TTTAACTTTA AGAAGGAGAT ATACATATG 89

56

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 51 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Lactobacillus casei

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AAAGTCTGT CAATTTTGT TCGGCGAAT GATAATGTGT TATACTCACA A 51

57

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 126 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Lactobacillus amylovorus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GCAAAAAAAT TTTCGATTTT TATGAAACCG GTTGCAAGA AGTTAGCAAA AATATATAAT 60
TTCTTTTGAA ATTGTTCACT TGGCCAAGCT GCAGTTTCAA TATTTTAATA AAGGGGCCAG 120
TAAAAA 126

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Lactobacillus pentosus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:
 AGAAAGCGTT TACAAAATA AGCCAATGCC GCTGTAATCT TAC
 43
 (2) INFORMATION FOR SEQ ID NO: 10:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: cDNA
 (v) FRAGMENT TYPE: N-terminal
 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:
 GATCATATGC GACCCCTCTGG GAGAAAATCC
 30
 (2) INFORMATION FOR SEQ ID NO: 11:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 28 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: cDNA

(v) FRAGMENT TYPE: C-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GATCTGCAGC TACTCGTCCT CCTGGAAG

28

(2) INFORMATION FOR SEQ ID NO: 12:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GTCCTGCTTT CTGGGCTCGC TCAG

24

(2) INFORMATION FOR SEQ ID NO: 13:

60

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (v) FRAGMENT TYPE: internal
- (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CTCAAACTG GCGTGGGC C

21

(2) INFORMATION FOR SEQ ID NO: 14:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 34 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (v) FRAGMENT TYPE: N-terminal
- (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GCGAATCTT ATCGGACCCT CTGGGAGAAA ATCC

34

(2) INFORMATION FOR SEQ ID NO: 15:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(v) FRAGMENT TYPE: C-terminal

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GGCTGCAGCT ACTCGTCCTC CTGGAAG

27

61

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 118 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GGGAATTC TT ATGAGAAGCA AAAAATTGTG GATCAGCTTG TTGTTTGGGT TAACGTTAAT 60

CTTTACGATG GCATTTCAGCA ACATGAGACG CCTCCGGCGA CCTCTGGGA GAAAATCC 118

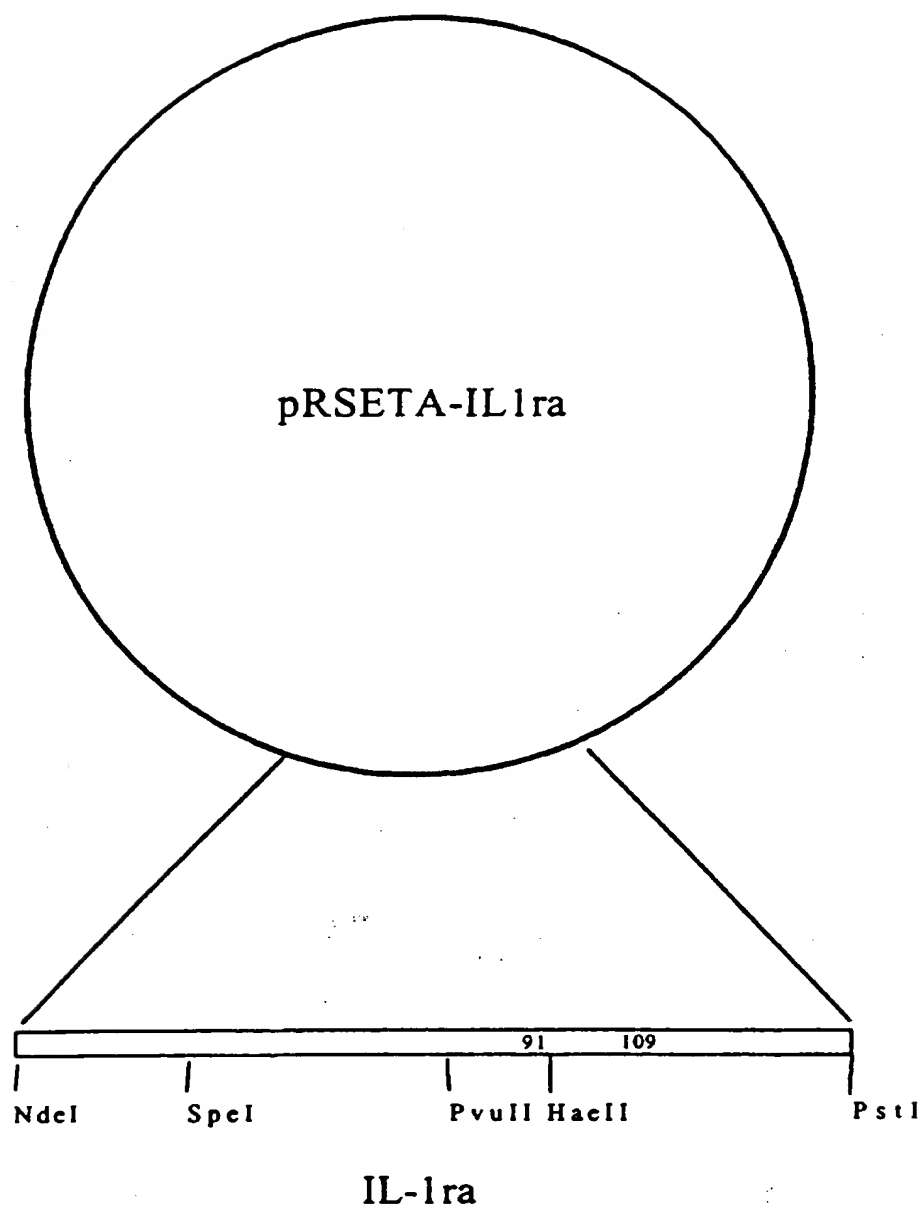
CLAIMS

1. Pharmaceutical compositions containing, as the active principle, engineered microorganisms expressing non-vaccinogenic pharmacologically active recombinant therapeutic proteins, wherein said microorganism is not *Salmonella* species.
2. Compositions according to claim 1 wherein said microorganisms are bacteria.
3. Compositions according to claim 2 wherein said bacteria are selected from the group consisting of *Lactobacillus*, *Escherichia coli* and *Bacillus*.
4. Compositions according to claim 3 wherein said bacteria are *Bacillus subtilis*.
5. Compositions according to anyone of claims 1 to 4 wherein said therapeutic protein is selected from the group consisting of cytokines, cytokine antagonists, growth hormone, trypsin inhibitors, interferons.
6. Compositions according to claim 5 wherein said therapeutic protein is selected from IL-1ra or mutants thereof, IL-10, interferons, and 1-antitrypsin.
7. Engineered microorganisms expressing non-vaccinogenic, pharmacologically active recombinant therapeutic proteins for use as therapeutic agents.
8. Use of engineered microorganisms expressing non-vaccinogenic, pharmacologically active recombinant therapeutic proteins for the preparation of medicaments for the treatment of diseases which are cured or alleviated by said therapeutic recombinant protein.

1/10

FIGURE 1

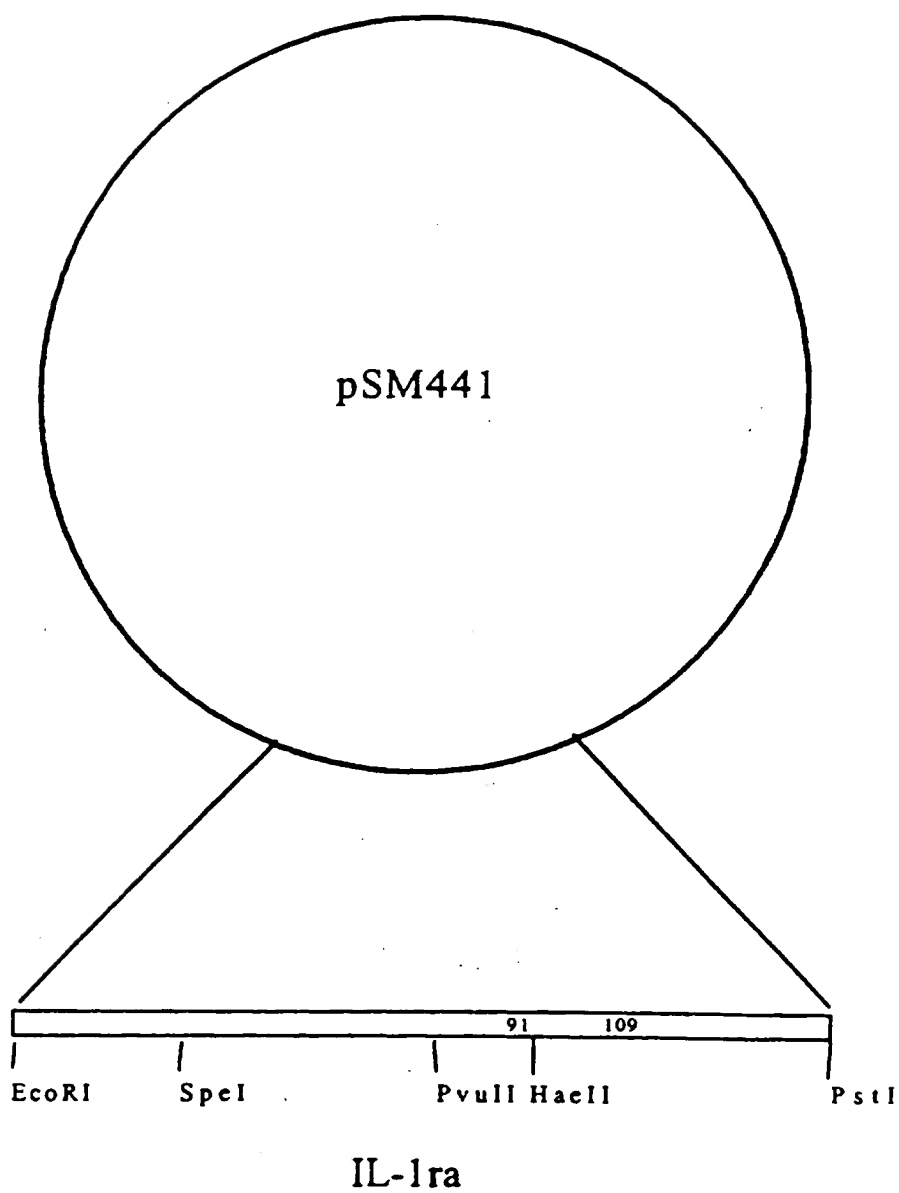
MAP OF THE EXPRESSION PLASMID pRSETA-IL1ra



2/10

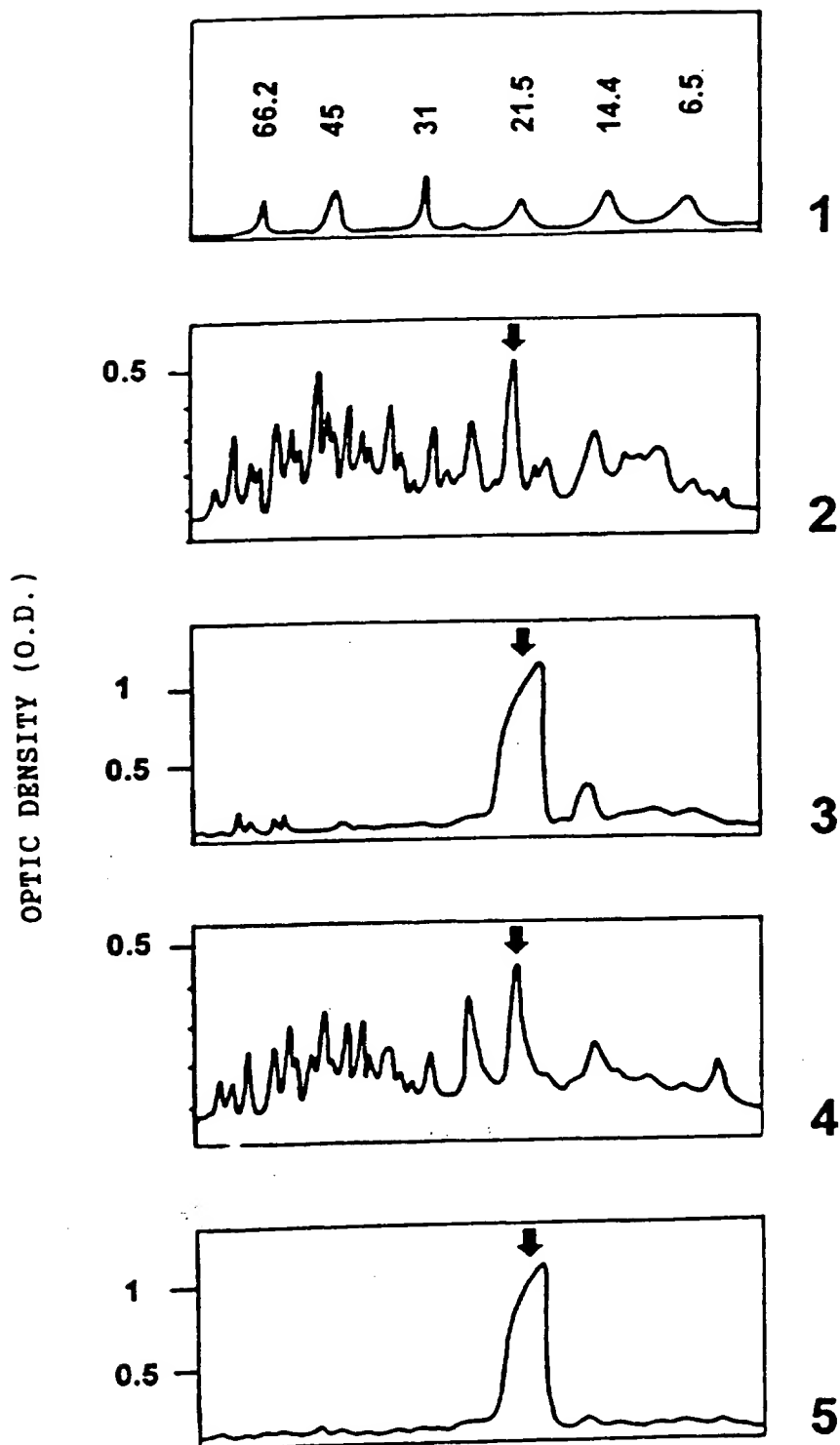
FIGURE 2

MAP OF THE EXPRESSION PLASMID pSM441



3/10

FIGURE 3



4/10

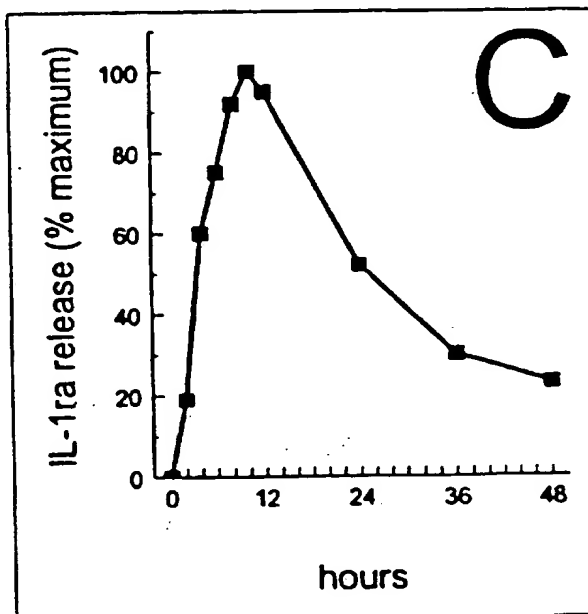
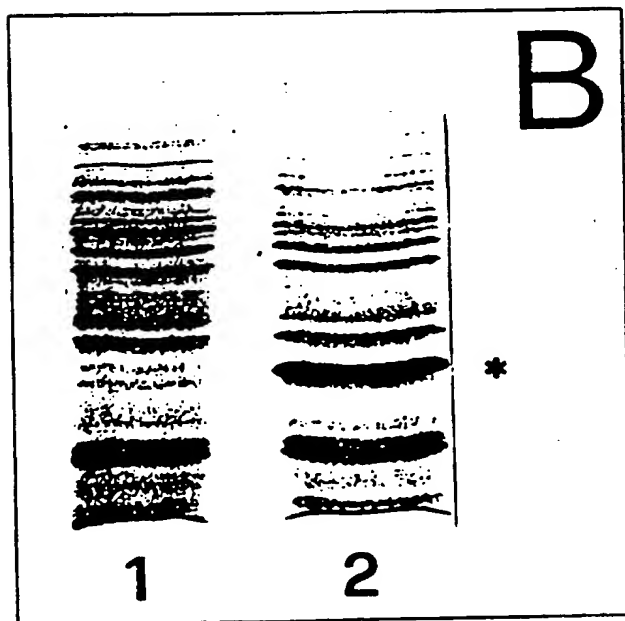
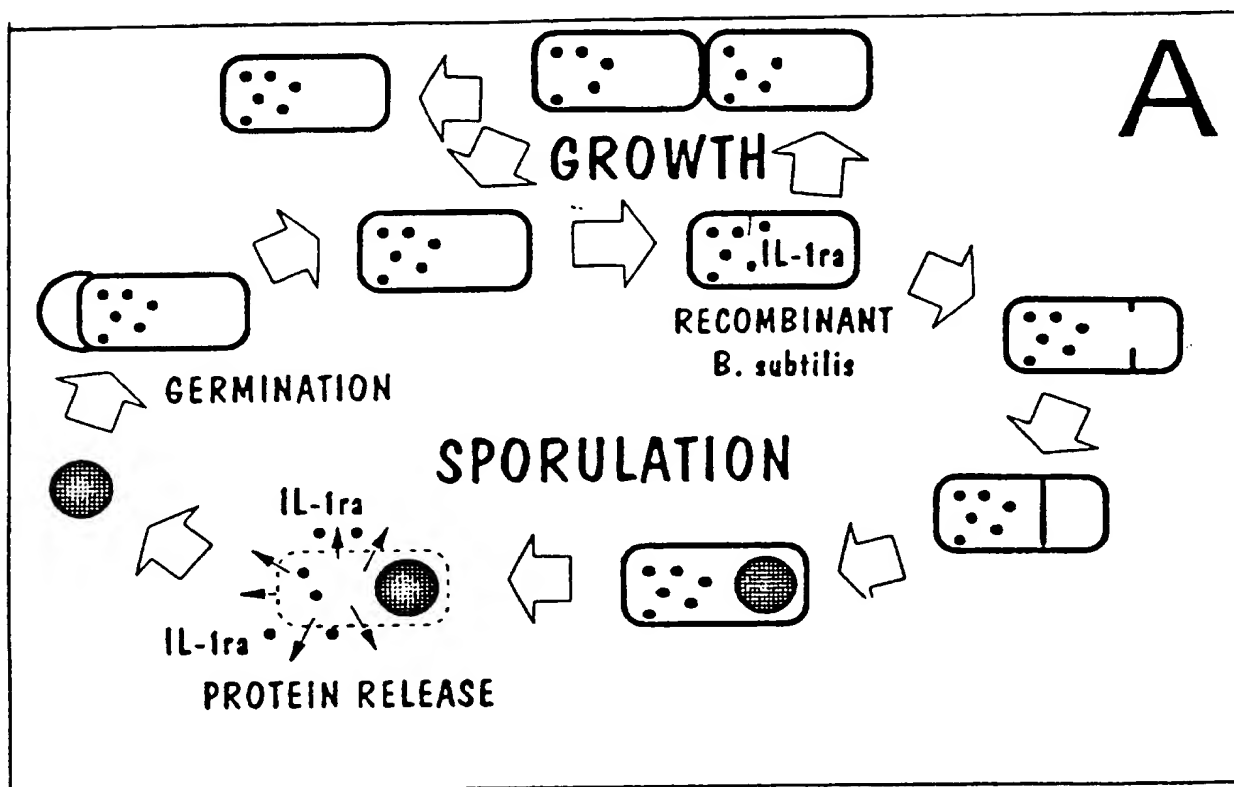
NUCLEOTIDE AND AMINO ACID SEQUENCE OF IL-1ra

1	ATGGAATCTGCAGAGGCCCTCCGAGTCACCTAATCACTCTCCTCCTCTTCTCTGTTCCAT	
11	M E I C R G L R S H L I T L L L F L F H	
61	TCAGAGACGATCTGCCGACCCCTCTGGAGAAAATCCAGCAAGATGCAAGCCTTCAGAATC	
21	S E T I C R P S G R K S S K M Q A F R I	
121	TGGGATGTTAACCAAGAGACCTTCTATCTGAGGAACAACCACTAGTTGCTGGATACTTG	
41	W D V N Q K T F Y L R N N Q L V A G Y L	
181	CAAGGACCAAAATGTCAATTTAGAAGAAAAGATAGATGTGGTACCCATTGAGCCTCATGCT	
61	Q G P N V N L E E K I D V V P I E P H A	
241	CTGTCTTTGGGAATCCATGGAGGAAGATGTGCCTGTCTGTGTCAAGTCTGGTGATGAG	
81	L F L G I H G G K M C L S C V K S G D E	
301	ACCAGACTCCAGCTGGAGGCAGTTAACATCACTGACCTGAGCGAGAACAGAAAGCAGGAC	
101	T R L Q L E A V N I T D L S E N R K Q D	
361	AAGCGCTTCGCCCTTCATCCGCTCAGACAGTGGCCCCCACCACCAAGTTTGTAGTCTGCCGCC	
121	K R F A F I R S D S G P T T S F E S A A	
421	TGCCCCGGTTGGTTCTCTGCACAGCGATGGAAGCTGACCAAGCCCGTCAGCCTCACCAAT	
141	C P G W F L C T A M E A D Q P V S L T N	
481	ATGCCTGACGAAGCGTCATGGTCACCAAAATTTCTACTTCCAGGAGGACGAG	531
161	M P D E G V M V T K F Y F Q E D E	177

FIGURE 4

5/10

FIGURE 5



6/10

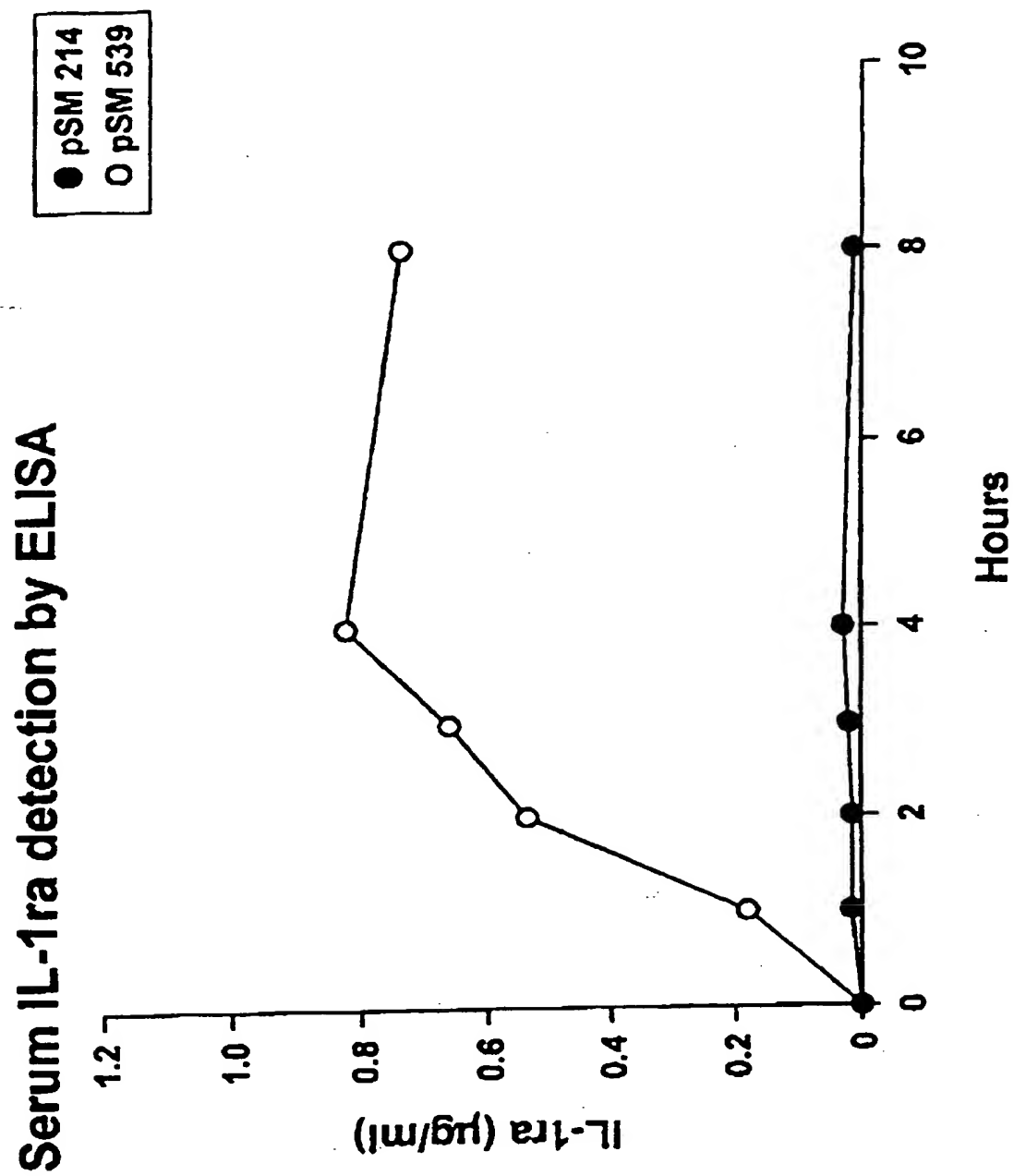


FIGURE 6

7/10

Serum IL-1ra detection by BIAcore

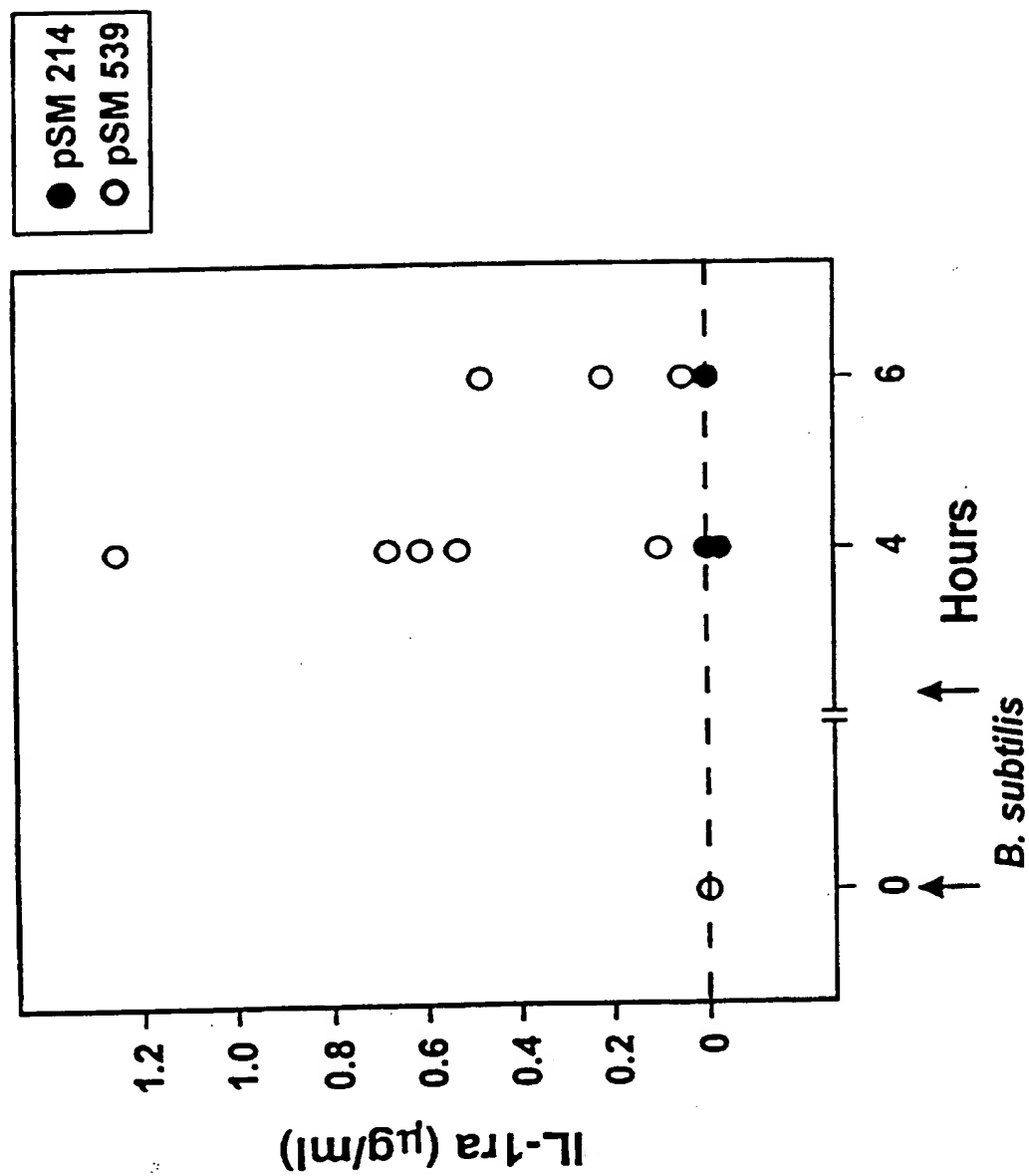


FIGURE 7

8/10

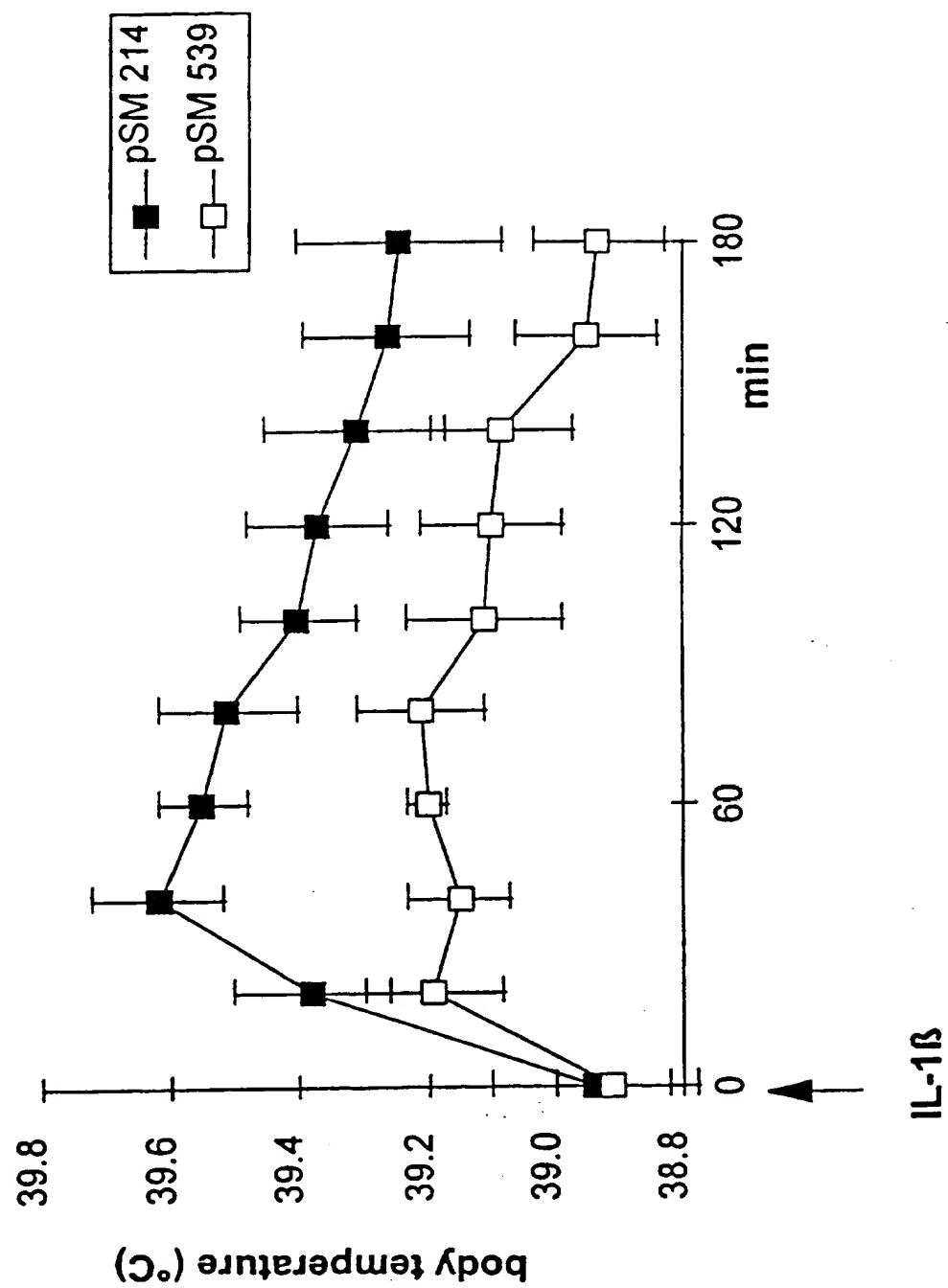


FIGURE 8

9/10

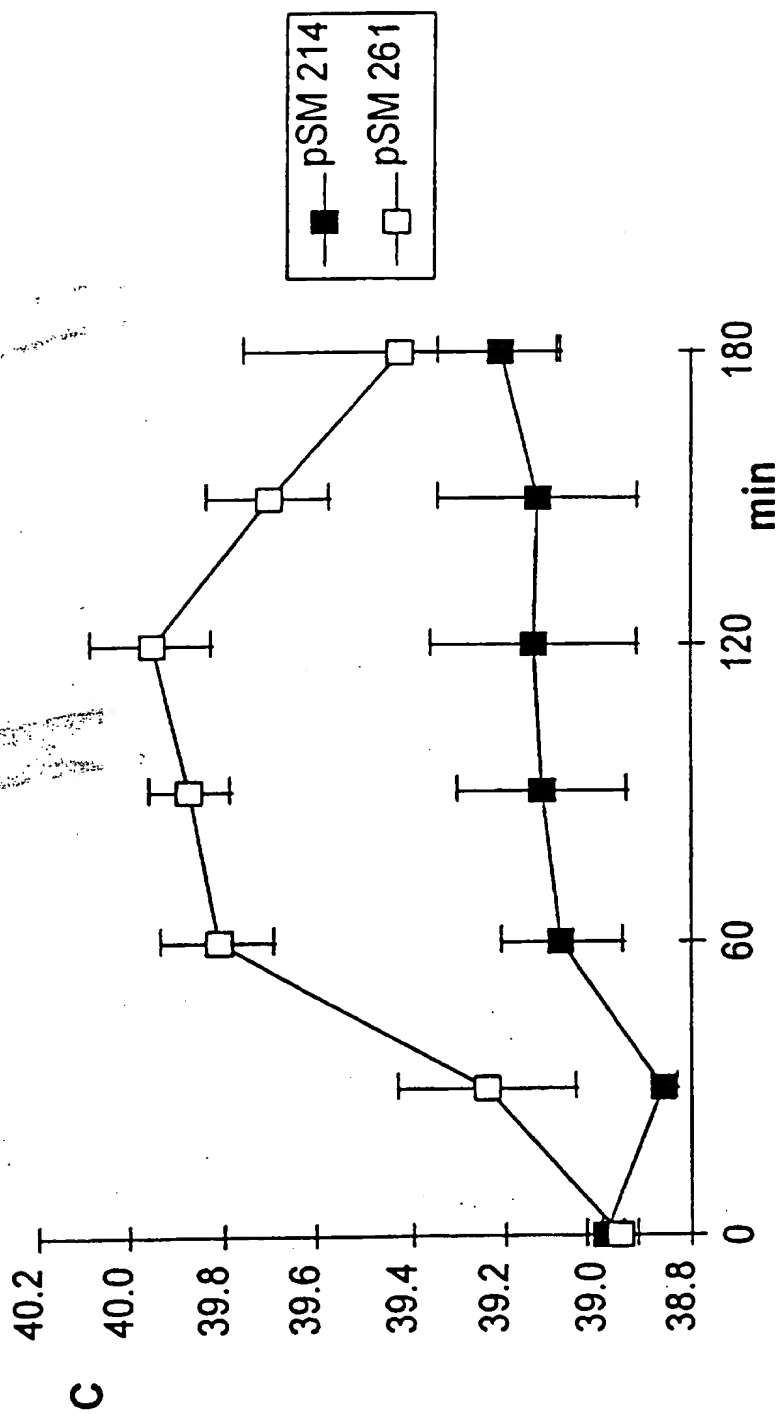


FIGURE 9

10/10

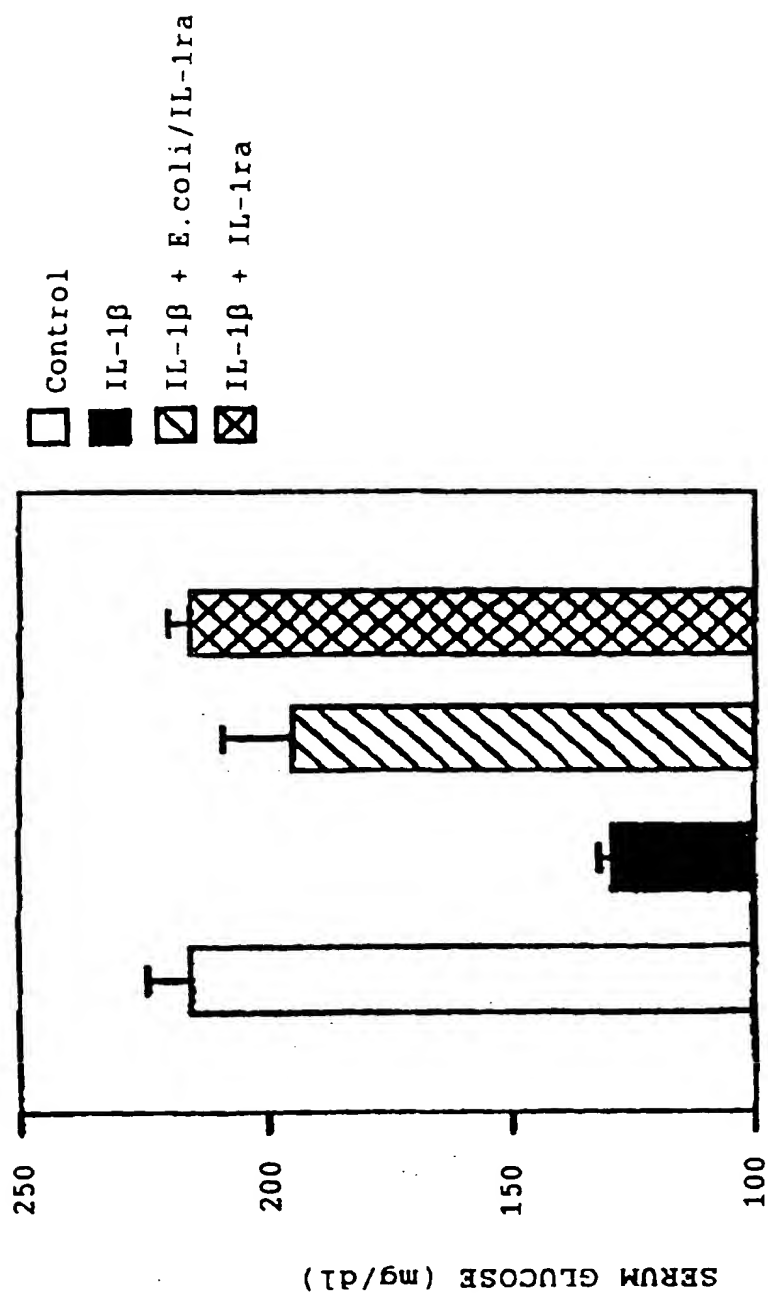


FIGURE 10

INTERNATIONAL SEARCH REPORT

International Application No

PC1/EP 95/03921

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/75 C12N15/74 C12N15/70 A61K47/48 A61K35/74
C07K14/54

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF IMMUNOLOGY, vol. 148, no. 4, 15 February 1992 BALTIMORE US, pages 1176-1181, M.J. CARRIER ET AL 'Expression of human IL-1beta in Salmonella typhimurium. A model system for the delivery of recombinant therapeutic proteins invivo' cited in the application see the whole document ---	1,2,5,7, 8
X	EP,A,0 195 672 (MATSUSHIRO, AIZO) 24 September 1986 see claims --- -/--	1-3,7,8

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

& document member of the same patent family

Date of the actual completion of the international search

13 February 1996

Date of mailing of the international search report

05.03.96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Le Cornec, N

INTERNATIONAL SEARCH REPORT

International Application No

PL 1/EP 95/03921

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HUMAN GENE THERAPY, vol. 5, no. 7, July 1994 pages 809-820, K.L. ELKINS ET AL 'In vivo delivery of Interleukin-4 by a recombinant vaccinia virus prevents Tumor development in mice' see the whole document ---	1,5,7,8
A	MICROBIOLOGICAL REVIEWS , vol. 57, no. 1, March 1993 pages 109-137, M. SIMONEN ET AL 'Protein secretion in Bacillus species' cited in the application ---	
A	IMMUNOLOGY TODAY, vol. 12, no. 11, 1991 CAMBRIDGE GB, pages 404-410, C.A. DINARELLO ET AL 'Blocking IL-1: Interleukin-1 receptor antagonist in vivo and in vitro' cited in the application -----	

INTERNATIONAL SEARCH REPORT

Int. Application No.

PCT/EP 95/03921

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-195672	24-09-86	AU-B- 592328	11-01-90
		AU-B- 5493786	25-09-86
		CA-A- 1321962	07-09-93
		DE-A- 3681946	21-11-91
		JP-A- 62000025	06-01-87
		KR-B- 9403689	27-04-94
		SU-A- 1720493	15-03-92
		US-A- 5306639	26-04-94
		US-A- 5290916	01-03-94

THIS PAGE BLANK (USPTO)